

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 37/38, 37/24	A1	(11) International Publication Number: WO 91/16922 (43) International Publication Date: 14 November 1991 (14.11.91)
(21) International Application Number: PCT/US91/03162 (22) International Filing Date: 7 May 1991 (07.05.91) (30) Priority data: 520,703 8 May 1990 (08.05.90) US (60) Parent Application or Grant (63) Related by Continuation US 520,703 (CIP) Filed on 8 May 1990 (08.05.90) (71) Applicant (for all designated States except US): UNIVERSITY OF MEDICINE & DENTISTRY OF NEW JERSEY [US/US]; 110 Administration Complex, 30 Bergen Street, University Heights, Newark, NJ 07101-3000 (US).		(72) Inventors; and (75) Inventors/Applicants (for US only) : CAMPBELL, Robert, K. [US/US]; 155 W. Franklin Avenue, Pennington, NJ 08854 (US). MOYLE, William, R. [US/US]; 952 River Road, Piscataway, NJ 08854 (US). (74) Agent: MUCCINO, Richard, R.; P.O. Box 1267, Princeton, NJ 08542 (US). (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US. Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: ANALOGS OF GLYCOPROTEIN HORMONES HAVING ALTERED IMMUNOLOGICAL CHARACTERISTICS, EFFICACY AND/OR RECEPTOR SPECIFICITY (57) Abstract <p>The invention relates to modified chorionic gonadotropin (CG) <i>alpha</i>, <i>beta</i>-heterodimeric polypeptides comprising a glycoprotein hormone <i>alpha</i>-subunit polypeptide and a non-naturally occurring <i>beta</i>-subunit polypeptide. The modifications in the <i>beta</i>-subunit polypeptide involve the deletion, addition, or substitution of one or more amino acid residues. The modified heterodimeric polypeptides of the invention have different properties compared to native hCG. Certain <i>alpha</i>, <i>beta</i>-heterodimeric polypeptides bind to LH and FSH receptors and stimulate steroidogenesis in testicular and ovarian cells. Other <i>alpha</i>, <i>beta</i>-heterodimeric polypeptides bind to LH receptors but have lower efficacy than hCG to stimulate steroidogenesis in testicular and ovarian cells.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TC	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

ANALOGS OF GLYCOPROTEIN HORMONES HAVING ALTERED IMMUNOLOGICAL CHARACTERISTICS, EFFICACY AND/OR RECEPTOR SPECIFICITY

5

FIELD OF THE INVENTION

10 The invention relates to glycoprotein hormone
analogs and other proteins, their methods of use and
other applications. The molecules of the invention are
analogs or derivatives of naturally occurring vertebrate
glycoprotein hormones and DNA molecules encoding them.
15 The naturally occurring hormones are typically
constituted of an alpha and beta subunit. Amino acid
sequences of naturally occurring alpha and beta-subunits
from several different species are presented in TABLE I.
Monomeric forms of both subunits as well as heterodimers
and homodimers are known to occur. In the molecules of
20 the invention the chemical structure of the beta-subunit
or the alpha-subunit or both may be different from that
of the native hormones. The glycoprotein hormone analogs
of the invention also differ from the naturally occurring
hormones in their biological, physiological,
25 pharmaceutical and/or immunological characteristics.
Observations that many different changes can be made in
the subunits of naturally occurring glycoprotein hormones
which can selectively alter specific properties of the
molecules has led to increased knowledge of hormone
30 structure. The hormone derivatives of the invention are
useful as tools with which various endocrine and
metabolic functions can be manipulated and dysfunctions
can be treated. One aspect of the invention relates to
the control of reproduction in mammals.

35

The hormones of the invention are useful in
numerous applications including therapeutic,
immunological, diagnostic, and other uses. Certain of
the hormones of the invention are capable of altering the

reproductive process in living organisms such as mammals and other vertebrates. Certain of the hormones promote fertility; others are contragestives. The various aspects of the invention are discussed in full detail hereinafter. The hormones of the invention may be produced by genetic engineering, chemical synthesis and/or a combination of such methods. The glycoprotein hormones of the invention contribute to fulfilling an important and actual need in the relevant field of art.

BACKGROUND OF THE INVENTION

The glycoprotein hormones include the gonadotropins and thyrotropins. In humans these are chorionic gonadotropin (CG), luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH). They are a family of heterodimeric proteins which share a common alpha-subunit but differ in their hormone-specific beta-subunits. Gonadotropins such as CG, LH and FSH play a major role in the reproductive process, while TSH is important for thyroid function. Owing to the importance of these hormones in endocrine and metabolic function, there has been considerable interest in characterizing the relationship between hormone structure and function.

While primary structures of many glycoprotein hormones have been known for many years, the relationship between hormone structure and function has been only partially characterized in spite of many studies involving chemical modifications (reviewed in 1), deglycosylation (1-4), immunological characterization (5-8), and use of peptide analogs (9-12). The beta-subunit specifies the activity of the hormones, however, neither free subunit has much biological activity (1). The reason the alpha, beta-heterodimer is the most active form is not known. Its activity might be the result of a

unique conformation of one or both subunits which forms after they combine (13) and/or it might be that portions of both subunits interact directly with the receptor (9, 10). The observation that some antibodies bind to epitopes on the hCG beta-subunit only when it is associated with the alpha-subunit suggests that the conformation of the beta-subunit is altered when it combines with the alpha-subunit (14). Residues responsible for the binding of these antibodies have been identified and include those between Cys-38 and Cys-57 (15), a region of the molecule which has been proposed to interact with the receptor (10). Similarly, some monoclonal antibodies and polyclonal antisera to the alpha-subunit (16) have higher affinity for the alpha, beta-heterodimer than for the free alpha-subunit, suggesting that the conformation of the alpha-subunit is altered when it combines with the beta-subunit. Evidence that both subunits participate in binding to the receptor is based on the ability of synthetic alpha and beta-subunit peptides to inhibit hCG binding to receptors (9, 10) and on the abilities of both subunits to be cross-linked to the receptor (17-19). It appears that the alpha-subunit may have a different conformation in different hormones since anti-alpha-subunit antibodies have been found which bind to the alpha-subunit in the heterodimeric forms of some but not all human glycoprotein hormones (29).

The Asn-linked carbohydrate residues influence efficacy of the hormones. Deglycosylation by chemical (3, 4), enzymatic (20, 21), or genetic (22) procedures renders them less active in vitro. The reasons the carbohydrate residues are required, particularly those on the alpha-subunit at Asn-52 (22), are unknown. The carbohydrate residues may participate in cross-linking the hormone-receptor complex to other cell surface proteins (2, 23). They may also alter the conformation of the molecules (24) although this has been hard to demonstrate spectroscopically (25). The carbohydrates

may increase the flexibility of the hormones and facilitate a change in conformation which occurs after the hormones bind to the receptor (26). A report that Fab fragments of antibodies to hCG can convert the
5 deglycosylated hormone into an agonist (27) lends further support for the effects of the carbohydrates on the conformation of hCG. Asn-linked sugars also appear to have a role in dimer stability since it is more difficult to dissociate the dimer following deglycosylation (28)
10 and since deglycosylated subunits recombine faster than native alpha and beta-subunits (25).

There are two major barriers to the understanding of the relationship between glycoprotein
15 hormone structure and function. First, the tertiary structures of the hormones have not been determined. The hormones resisted crystallization and only recently have high quality crystals for x-ray diffraction studies of a glycoprotein hormone (hCG) been obtained (71, 72). In
20 addition, there is doubt regarding the locations of the disulfide bonds in the subunits (1, 2). Due to the difficulties anticipated in obtaining the tertiary structure of the carbohydrate chain, known to influence the activity of the hormone, even crystallographic and/or
25 NMR approaches may not guarantee the characterization of residues which interact with hormone receptors to induce a response.

The second major impediment to understanding
30 the relationship between structure and function has been the difficulty of preparing analogs. Until 1985 (30), the only means of preparing analogs was by chemical or enzymatic means. The limited numbers of residues which could be specifically modified and the difficulty of
35 removing particular N-linked sugar chains curtailed progress substantially.

Present day recombinant DNA technology permits the production of a much wider range of analogs. Several

-5-

glycoprotein hormones have now been expressed from DNA introduced into mammalian cells in culture, and analogs of some have also been expressed (22, 30-36).

5 The present invention utilizes DNA mutagenesis and gene expression to produce glycoprotein hormone analogs. These analogs have enabled identification of residues important for receptor binding and specificity, subunit interactions, and antibody binding.

10 It has been discovered that a wide variety of changes can be made in these hormones. For example, chimeric subunits can be created which combine specific characteristics of each parent molecule such as receptor
15 or antibody binding (TABLES II, III, IV, VI, XIII and FIGURE 11). Moreover, deletions of certain regions can result in molecules having decreased efficacy (TABLE VII and FIGURE 14).

20 Identification of amino acid residues in the hormones that contribute to their biological, physiological, pharmacological, and/or immunological characteristics (FIGURES 7-12, 14) has enabled the
25 production of a variety of useful analogs. The invention provides for compounds useful for manipulation of gonad or thyroid function. Among these uses are the induction of fertility in male and female mammals, promotion of female infertility, contragestion and the in vitro and in vivo control of reproductive capacity in animals.

30

BACKGROUND ART

35 The glycoprotein hormones have been studied extensively. There has been considerable interest in the structure/function relationships of these molecules. The general references listed below reflect the current state of knowledge in this area.

PIERCE, J.G. and PARSONS, T.F., Ann. Rev. Biochem. 50, 465 (1981). This extensive review article summarizes the results of various chemically and enzymatically derived analogs of the naturally occurring glycoprotein hormones to discern the relationship between their structure and function. The authors conclude that much remains to be known about the interaction of the glycoprotein hormones with their receptors.

RYAN, R.J. et al., Recent Progress Hormone Res. 43, 383 (1987). RYAN, R.J. et al., "The Glycoprotein Hormones: Recent Studies of Structure-Function Relationships", FASEB Journal 2, 2661 (1988). These articles summarize studies employing synthetic peptides to study glycoprotein hormone structure and function.

KEUTMANN, H.T. et al., Proc. Nat'l. Acad. Sci. USA 84, 2038 (1987). This work concludes that the intercysteine 38-57 loop sequence in the beta-subunit of hCG and hLH is a determinant for expression of biological activity in these molecules.

SCHNEIDER, A.L. et al., Biochemistry 27, 666 (1988). This work concludes that hFSH beta-subunit sequences 33-52 are important in receptor binding, and that the sequence TRDL (34-37) is of particular importance.

An example of work identifying the role of the subunits in determining binding specificity is Strickland, T.W. and Puett, D., "Contribution of Subunits to the Function of Luteinizing Hormone/Human Chorionic Gonadotropin Recombinants", Endocrinology 109, 1933 (1981). Hybrid recombinants prepared from bovine and porcine LH and human CG were used. It was observed that both the alpha and beta-subunits were found to contribute to the properties of the recombinant in several in vitro

systems. In each case, the recombinant behaved most like the hormone from which the beta-subunit was derived.

For a general reference on reproductive endocrinology, see Yen, S.S.C. and Jaffe, R.B., "Reproductive Endocrinology: Physiology, Pathophysiology and Clinical Management", 2nd edition, W.B. Saunders Company, Philadelphia (1986), hereinafter referred to as "Yen and Jaffe". Chapter 19 of this text reviews both the mechanisms involved in fertilization and the clinical problems of infertility. This text illustrates uses of glycoprotein hormones for induction of fertility.

BRIEF DESCRIPTION OF THE FIGURES AND TABLES

FIGURE 1 depicts the nucleotide sequence and encoded amino acid sequence of the hCG beta-subunit cDNA along with several restriction endonuclease recognition sites.

FIGURE 2 depicts the nucleotide sequence and encoded amino acid sequence of the hCG alpha-subunit cDNA along with several restriction endonuclease recognition sites.

FIGURE 3 depicts the sequences DNA oligonucleotides used to construct plasmids pKBMt and pKBM.

FIGURE 4A depicts the nucleotide sequence and encoded amino acid sequence of the analog hCG-beta, which contains two silent mutations that eliminate restriction endonuclease recognition sites present in the hCG beta-subunit cDNA; and FIGURE 4B depicts the DNA oligonucleotides used to construct this analog by cassette mutagenesis.

FIGURE 5 depicts the nucleotide sequences of DNA oligonucleotide cassettes used to construct analogs F8 (see TABLE VII), B9 and B11 (see TABLE II).

5 FIGURE 6 depicts the nucleotide sequence and encoded amino acid sequence of the synthetic bovine alpha-subunit gene, along with several restriction endonuclease recognition sites.

10 FIGURE 7 illustrates human alpha-subunit residues critical for antibody binding.

 FIGURE 8 illustrates hCG beta-subunit residues critical for antibody binding.

15 FIGURE 9 is a dose response curve of LH receptor binding by hCG/hFSH chimeric beta-subunits co-expressed in COS7 cells with human alpha-subunit, compared with hCG and hFSH.

20 FIGURE 10 is a dose response curve of FSH receptor binding by hCG/hFSH chimeric beta-subunits co-expressed in COS7 cells with human alpha-subunit, compared with hCG and hFSH.

25 FIGURE 11 is a dose response curve of LH receptor mediated steroidogenic activity of hCG/hFSH chimeric beta-subunits co-expressed in COS7 cells with human alpha-subunit, compared with hCG and hFSH.

30 FIGURE 12 is a dose response curve of FSH receptor mediated steroidogenic activity of hCG/hFSH chimeric beta-subunits co-expressed in COS7 cells with human alpha-subunit, compared with hCG and hFSH.

35 FIGURE 13 is a dose response curve of LH receptor binding by an hCG beta-subunit deletion analog (F8) co-expressed in C127 cells with human alpha-subunit, compared with hCG.

FIGURE 14 is a dose response curve of LH receptor mediated adenylate cyclase activity of an hCG beta-subunit deletion analog (F8) co-expressed in C127 cells with human alpha-subunit, compared with hCG.

TABLE I depicts amino acid sequences of several naturally occurring glycoprotein hormone alpha-subunits and beta-subunits.

TABLE II depicts amino acid sequences of several hCG/hFSH beta-subunit chimeras.

TABLE III depicts amino acid sequences of several hCG/hTSH beta-subunit chimeras.

TABLE IV depicts amino acid sequences of several hCG/bLH beta-subunit chimeras.

TABLE V depicts amino acid sequences of several hCG/eLH beta-subunit chimeras.

TABLE VI depicts amino acid sequences of several hCG/hLH beta-subunit chimeras.

TABLE VII depicts amino acid sequences of several beta-subunit deletion mutants.

TABLE VIII depicts amino acid sequences of several other beta-subunit analogs.

TABLE IX depicts amino acid sequences of several beta-subunit analogs.

TABLE X depicts examples of several beta-subunit/fusion proteins.

TABLE XI illustrates differences in monoclonal antibody binding among chimeric human/bovine beta-

subunits co-expressed with hCG beta-subunit compared with human and bovine alpha-subunits co-expressed with hCG beta-subunit.

5 TABLE XII illustrates the ability of membrane bound hCG-beta/VSV-G fusion protein to combine with the hCG alpha-subunit and be recognized by monoclonal antibodies to beta-subunit (B105), alpha-subunit (A111) and the alpha, beta-heterodimer (B109).

10 TABLE XIII illustrates monoclonal antibody binding to chimeric hFSH/hCG beta-subunits.

15 TABLE XIV illustrates LH receptor binding by alpha, beta-heterodimers containing chimeric alpha-subunits or alpha-subunit deletions.

20 TABLE XV illustrates monoclonal antibody binding to hCG-beta/VSV-G fusion proteins on stably transfected COS7 cells compared with COS7 cells stably transfected with hCG beta-subunit.

25 SUMMARY OF THE INVENTION

30 The invention provides protein hormone analogs which are "engineered" such that they are capable of controlling various hormone-regulated physiological functions in vertebrates of both genders. In particular, the invention relates to the glycoprotein hormones and their individual subunits. The heterodimeric forms of the hormones are thought to be the most active. The four human hormones belonging to this group are chorionic gonadotropin (CG), luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH).

35

-//-

In accordance with the invention, the terminology "hormone analogs", "glycoprotein hormone analogs" or "variants" is to be understood in the broadest manner. Within the description the term "chimera" is used to designate a hormone analog which contains amino acid sequences derived from two or more different glycoprotein hormones. These chimeras can be constructed from proteins with low sequence homology. For example, beta-subunit chimeras can be made from hCG and hFSH beta-subunits in regions of very low sequence homology (TABLE II). These chimeras, described in detail below, exhibit useful properties such as the ability to bind and stimulate both LH and FSH receptors. In some instances comparatively small changes, such as modification of a single amino acid in the hormone sequence, cause significant alteration of the activity as compared to the native hormone.

The analogs contemplated by the invention include molecules which have novel biological, physiological, pharmacological and/or immunological (hereinafter "biological") activities and molecules that are useful intermediates for such active molecules. Within the scope of the invention are also modified alpha and/or beta-subunits, not only the coupled dimers. These subunit analogs may be used in the monomeric ("free subunit") form. The modified subunits may also be coupled with an appropriate other subunit. Further, as will become apparent from the description, the hormone analogs of the invention need not be constituted from the same animal species, they can be prepared using portions or combinations of the hormones obtained from any vertebrate, i.e., "cross-species" chimeras (for examples see TABLES IV, V, and IX). Further, within the scope of the invention are applications and uses in the human field, in the veterinary field, and other fields of use, including applications in other vertebrates. As further described, some analogs of the invention have the

property of influencing fertility (e.g., promoting or inhibiting) in humans and animals.

5 In accordance with the invention, particular domains of amino acids are identified to which specific physiological functions or other biological activities can be attributed (FIGURES 7-12). The invention encompasses a wide variety of variants of the alpha and beta-subunit which in the dimer or monomer form exhibit
10 new or altered biological activities. It is contemplated that some analogs of the invention will be useful as agonists or antagonists which can act as receptors for one or more different glycoprotein hormones.

15 In another aspect of the invention, fusion proteins have been made between a glycoprotein hormone beta-subunit (hCG-beta) and another protein (TABLE X). Particularly there is described an hCG beta-subunit/VSV G-protein analog, in which amino acid residues from the
20 transmembrane and cytoplasmic domains of the vesicular stomatitis virus G-protein have been inserted in the hCG-beta-subunit downstream of Cys-110 at hCG-beta using the SmaI restriction endonuclease site at residues 135-137 (TABLE XII). It is contemplated that this analog will be
25 useful as a screening system for analysis of mutagenesis products and as an immunogen which can be introduced into an animal or human attached to the cells or organisms (such as a virus) producing it, thereby eliminating the need to isolate and purify the protein itself.

30 The invention provides for proteins in which amino acid residues from a glycoprotein hormone subunit (or subunits) are inserted into another protein. For example, it is contemplated that hormone residues
35 involved in antibody recognition may be transferred to a non-glycoprotein hormone (e.g., lysozyme) and the resulting protein used as an immunogen to elicit an immune response against selected regions of a glycoprotein hormone. Another aspect of the invention

provides for the design of peptides useful as immunogens which induce an immune response capable of specifically inhibiting the action of one or more glycoprotein hormones. It also provides for the design of peptides
5 useful as receptor agonists or antagonists.

It is also contemplated that regions of a glycoprotein hormone subunit, for example hCG-beta 39-56 or hFSH-beta 33-50, could be replaced with a sequence
10 from a non-glycoprotein hormone and used as an antigen to develop antisera or antibodies to the inserted sequence.

One aspect of the invention provides beta-subunit chimeras that are capable of directing hormone
15 binding to both LH and FSH receptors. These chimeras may be derived from human glycoprotein hormones (e.g., hCG/hFSH or hLH/hFSH) or non-human glycoprotein hormones (e.g., bovine LH/bovine FSH or equine LH (or CG)/equineFSH). It is contemplated that these chimeras
20 will be useful for the treatment of infertility in men and women and the promotion of fertility in male and female animals.

The invention also provides alpha-subunit
25 analogs. Among these are chimeric alpha-subunits comprised of sequences from alpha-subunits from different species (TABLE IX). These analogs exhibit novel immunological properties. It is contemplated that these analogs will be useful immunogens. Bovine/human alpha-
30 subunit chimeras have enabled the identification of particular regions of the subunit which are involved with antibody binding (FIGURE 7), and may be used to restrict or alter the immune response to an immunogen.

35 Cross-species beta-subunit chimeras such as hCG/bovine LH (TABLE IV) or bovine LH/equine LH are also provided by the invention. Likewise, cross-species chimeras derived from other vertebrates are contemplated. It is contemplated that these chimeras will have

veterinary applications and also be useful for mapping regions of the hormones involved in receptor binding and biological efficacy.

5 Another class of chimeras provided by the invention includes gonadotropin/thyrotropin analogs (TABLE III). These analogs are expected to be useful for manipulating gonad or thyroid function. They will also be useful for identifying amino acid residues responsible
10 for hormone function. This information will facilitate the production of improved hormone antagonists, agonists, partial agonists and analogs that combine agonist/antagonist activity to different receptors.

15 Some constructs provided by the invention will enable development of antibodies to specific domains on glycoprotein hormones. It is expected that these antibodies will be hormone-specific, and thus will not exhibit undesirable binding to other hormones. Such
20 constructs include alpha and beta-subunit chimeras and analogs in which carbohydrate groups have been introduced to block antigenicity of selected regions of the hormones. Other analogs are contemplated in which known T-cell epitopes are placed into the hormones to
25 facilitate their immunogenicity. Two utilities are anticipated. In the first, it is contemplated that these constructs will be useful as immunocontragestive vaccines. These immunogens are contemplated to be useful in this regard in that the antibodies generated will be
30 high titer and specific for particular parts of the hCG molecule. The hCG-antibody complex elicited will prevent hCG from the placenta from stimulating the corpus luteum, thereby causing termination of pregnancy at an early stage. The second application contemplates controlling
35 the reproductive capacity of animals. More specifically, the immunogenic constructs of the invention are contemplated to be useful for immunocastration of steers, horses, dogs and cats and other vertebrates. Additionally, the suppression of the development of the

reproductive system of heifers is contemplated as a way to control heat in the feed lot.

5 The invention also provides constructs that are glycoprotein hormone receptor antagonists. It is contemplated that these antagonists will have several applications. In particular hormone antagonists can be used to terminate ectopic pregnancy, block the action of LH in polycystic ovarian disease, suppress gonadal
10 activity during cancer chemotherapy, or block the action of stimulatory immunoglobulins on the thyroid in Graves' disease.

15 In summary, the invention provides analogs of glycoprotein hormones which will have a variety of biological characteristics.

DETAILED DESCRIPTION OF THE INVENTION

20

 The invention includes three major embodiments. The first includes analogs of glycoprotein hormones capable of the induction of fertility in men, women and
25 animals of both genders. The second includes analogs which will function as immunocontragestive vaccines, immunocastration agents and suppressors of reproductive system development. Embodiment three includes antagonists capable of inhibiting hormone or receptor
30 activity.

INDUCTION OF FERTILITY IN WOMEN, MEN AND ANIMALS

35 Infertility is a significant clinical, social and human health problem. Many women who desire to become pregnant fail to develop a dominant follicle and trigger the hCG surge needed for ovulation. If an ovulation is caused by gonadotropin deficiency, such as in polycystic ovarian disease, follicular maturation and

ovulation can be induced by administration of human menopausal gonadotropin (hMG) followed by hCG. hMG is an extract prepared from the urine of post-menopausal women containing hLH and hFSH. It is sold commercially under the tradename Perganol(TM).

In women both hLH and hFSH are necessary for ovarian steroidogenesis, follicle maturation, and ovulation. During follicular development in the ovary, LH stimulates the cells surrounding the follicle and induces androgen production. Cells inside the follicle surrounding the oocyte are responsive to FSH and convert the androgens produced by the thecal and interstitial cells into estrogens.

Certain constructs of the invention are contemplated to be useful for the treatment of female infertility caused by gonadotropin deficiency. These molecules interact with LH and FSH receptors such that when administered to an infertile female, estrogen formation and follicular maturation should be induced. Thus, the invention discloses a protein which can replace Perganol (TM), hMG or other mixtures of hLH and hFSH in the treatment of infertility.

Preferred embodiments of the invention are chimeras in which residues near the carboxy-terminal region of hFSH are substituted for the corresponding region of hCG. These chimeras combine with the human alpha-subunit to form a dimer which has biological activity. Four specific chimeras are discussed below. The sequences of these constructs are shown in TABLE II and are denoted B11, B17, B18 and B19. All of these constructs combine with the alpha-subunit to form a heterodimer which can bind LH receptors and stimulate Leydig cell steroidogenesis (20) as well as bind FSH receptors and stimulate granulosa cell steroidogenesis (37). Antibody binding to these analogs has also been

characterized. Results of representative assays are indicated in TABLE XIII and FIGURES 9-12.

5 A preferred embodiment of the invention is construct B18. This construct is alternately designated beta-CF94-114, where C and F indicate the pattern of hCG-beta and hFSH-beta sequences, respectively, and the numbers indicate the first and last non-identical residues in each region of substitution. Construct B18
10 is also alternately designated DG'. In this construct, residues 88-108 of hFSH-beta replace residues 94-145 of hCG-beta. This construct combines with the alpha-subunit to form a heterodimer that can bind both LH and FSH receptors and stimulate steroidogenesis (FIGURES 11 and
15 12).

Other embodiments of the hCG/hFSH beta-subunit chimeras are constructs B11, B17, and B19. Construct B11, alternately designated beta-CF94-117 or DG, contains
20 residues 88-111 of hFSH-beta in place of residues 94-145 of hCG-beta. Construct B17, alternately designated beta-CFCF39-58/94-117 or ABCDG, contains residues 33-52 and 88-111 of hFSH-beta in place of residues 39-58 and 94-145 of hCG-beta, respectively. Construct B19, alternately
25 designated beta-CFCF39-58/94-113, 114Q or ABCDG', is a variant of construct B17 which combines chimeric and non-chimeric substitutions and a deletion. The amino acid sequence is the same as B17 except that a glutamine (Q) is substituted for glutamic acid (E) at position 114, and
30 the last three residues (115-117) are deleted.

In addition to these embodiments, it is contemplated that smaller regions of hFSH-beta such as the sequence DSDSTDCTVRGLGPSY (hFSH-beta residues 88-
35 103), TVRGLGPSY (hFSH-beta residues 95-103) or TVRGLG (hFSH-beta residues 95-100) could be inserted into appropriate regions of the hCG (or hLH) beta-subunit and that the resultant chimeric beta-subunit would direct binding of the alpha, beta-heterodimer to FSH receptors,

eliciting a biological response in addition to the molecule's native LH receptor binding activity and efficacy.

5 It is also contemplated that hCG-beta or hLH-beta sequences can be substituted at the carboxy terminal of hFSH, as for construct B25, and that smaller regions of hCG-beta can be substituted into hFSH-beta, as for
10 construct B26, and that these chimeric proteins would direct binding of the alpha, beta-heterodimer to LH receptors, eliciting a biological response. It is further contemplated that these various chimeras may exhibit differing potencies of activity at the LH and FSH
15 receptors, leading to the selection of one over the others for certain applications because of its higher or lower ratio of FSH to LH activity.

 The finding that the constructs of the invention containing hFSH-beta residues in place of hCG-beta
20 beta residues 94-145 will direct binding to LH and FSH receptors and stimulate steroidogenesis was an unexpected one. The prior art shows that other regions of the beta-subunit were thought to be important for receptor binding. Schneyer et al. (12) indicate that the TRDL
25 sequence of hFSH-beta (residues 34-37) are necessary for binding to FSH receptors. Subsequent studies by Santa Coloma et. al. (76) indicate that hFSH-beta residues 33-53 bind and stimulate FSH receptors. Keutmann et al.
30 (10) concluded that corresponding region of hCG-beta and hLH-beta, residues 38-57, were necessary for binding and stimulation of LH receptors. These authors also concluded that hCG-beta residues 93-101 were necessary for binding to LH receptors. If these regions of the
35 beta-subunit were important for receptor binding specificity, chimeric hCG/hFSH beta-subunits containing sequence alterations in these regions would exhibit altered binding specificity. This is not the case. Data obtained with constructs B9, B15, and B16 reflect this (FIGURES 9-12). Construct B15, alternately designated

-19-

CFC39-58 or ABC, contains hFSH-beta residues 33-52 in place of hCG-beta residues 39-59. The receptor binding activity of the alpha, beta-B15 heterodimer is virtually indistinguishable from hCG (FIGURES 9 and 10). Construct B9, alternately designated CFC94-97 or D, contains hFSH-beta residues 88-91 in place of hCG-beta 94-97 (residues corresponding to hCG-beta 98-100 are identical in hFSH-beta and hCG-beta). The alpha, beta-B9 heterodimer binds and stimulates LH receptors, but with about 30-fold less potency than hCG (FIGURES 9 and 11). The alpha, beta-B9 heterodimer exhibits no FSH receptor binding at the amounts tested (FIGURE 10). Construct B16, alternately designated CFCFC39-58/94-97 or ABCD, combines the substitutions present in B9 and B16. As for B9, the alpha, beta-B16 heterodimer is about 30-fold less active than hCG at LH receptors (FIGURES 9 and 11). It is no better than hCG in binding to FSH receptors (FIGURE 10).

It is further contemplated that the chimeric constructs of the invention would be useful for the enhancement of fertility in men and animals of male gender, i.e., to stimulate spermatogenesis. Induction of spermatogenesis requires both LH and FSH. LH stimulates testosterone production by Leydig cells of the testes. FSH and the high local concentrations of testosterone produced in response to LH stimulate the Sertoli cells and thereby induce spermatogenesis. For this reason, compounds that act on both FSH and LH receptors are desirable. Further, since it is known that hCG has a much longer half-life than FSH, it would be expected that hormone analogs containing hCG/hFSH-beta-subunit chimeras would have a longer biological half-life than hFSH. These analogs would be anticipated to be more efficacious than hCG or hFSH alone or mixtures of hCG and hFSH.

IMMUNOLOGICAL CONTROL OF THE REPRODUCTIVE PROCESS

It is contemplated that the glycoprotein hormone analogs of the invention will permit the

development of high titer specific antisera to hCG and thus be useful as long-term anti-fertility agents. The need for antigens capable of eliciting specific antisera to glycoprotein hormones for diagnostic immunoassays of the hormones and for the development of vaccines against hCG has long been recognized.

It is anticipated that the analogs of the invention which retain regions of tertiary structure present in hCG-beta will be considerably more potent than synthetic peptides in eliciting an immune response to specific domains of hCG and be more efficacious as an immunocontraceptive vaccine. Recent studies in which carbohydrates are added to proteins have demonstrated that it is possible to reduce their antigenicity (39). Thus, it is contemplated that the addition of carbohydrate residues to some regions of the hCG-beta-subunit will reduce the immunological cross-reactivity between analogs of the invention and the other glycoprotein hormones (in human these are LH, FSH, and TSH). A preferred embodiment of the invention is analog G9 (TABLE VIII). In this analog hCG beta-subunit residue 78 is replaced with another amino acid and residue 79 is replaced with either a serine or a threonine residue to create a glycosylation signal for Asn-77, thereby reducing the antigenicity of the major common epitope between hLH and hCG. It is not to be excluded that analogs of the invention may be immunogenic or be of increased immunogenicity when conjugated to an appropriate carrier, such as muramyl dipeptide (MDP), derivatives thereof or other suitable carriers. The carrier may or may not be immunogenic. The immunogenic analogs of the invention are formulated with a suitable vaccine adjuvant, as is known in the art. Methods of vaccination for humans and animals are known in the art.

The antigens of the invention will generate an immune response to specific domains of the hCG molecule. The antibodies generated will bind endogenous circulating

-21-

hCG produced by the placenta, blocking it from binding corpus luteum LH receptors with the result that the corpus luteum will stop secreting progesterone. Because of the need for ovarian progesterone in maintenance of early pregnancy, a lack of this steroid hormone will result in termination of the pregnancy.

It is also contemplated for the constructs of the invention to be useful for controlling the reproductive capacity and secondary sex characteristics of animals. One such use is as an immunological replacement for surgical castration of bulls. Bulls are castrated to prevent them from fighting and damaging each other. However, there are numerous problems which result from castration. There is a fairly high chance of infection and some morbidity. The resultant steer also does not eat regularly for a period after surgery and as a result, weight gain is retarded. Even after an animal has recovered from surgery, the rate of weight gain remains lower and the meat produced by the animal has a higher fat content. To compensate for these effects, farmers feed their cattle androgen supplements. The resultant increase in circulating androgen leads to faster weight gain and meat with a higher protein content. However, this practice has been found not to be totally acceptable. Constructs of the invention would be useful as immunogens for generating antibodies to specific structural domains of bovine LH. The antibodies would partially neutralize the hormone resulting in prevention of puberty but still provide sufficient circulating testosterone to have the positive effects on weight gain. Further, it can be seen that immunogens of the invention can be useful for suppressing the development of the reproductive system to control heifer heat in the feed lot, spay cats and dogs, preclude gelding of horses and other such applications.

Numerous other practical uses and applications of the immunogenic constructs are considered within the

scope of this invention. The generation of high titer immune responses to particular domains of glycoprotein hormones in which the antibodies produced are not cross-reactive to other glycoprotein hormone species permits
5 the control of the physiological function with which the particular glycoprotein hormone is associated.

HORMONE ANTAGONISTS

10 In accordance with the invention, constructs of the invention are useful for the non-surgical termination of pregnancy and ectopic pregnancy. Contragestion is accomplished by certain constructs of the invention which
15 block access of native hCG to the corpus luteum. These constructs exhibit hCG receptor binding specificity but a decreased efficacy of steroidogenesis induction. This decreased induction of progesterone formation results in inadequate levels for maintenance of the pregnancy.

20 A preferred specific embodiment of the hCG antagonists is construct F8 (TABLE VII). In this construct, residues 47-50 were deleted from native hCG-beta and residue 51 was changed from alanine (A) to proline (P). When combined with alpha-subunit this
25 construct has been shown to bind LH receptors (FIGURE 13) and stimulate hCG induced cyclic-AMP accumulation at approximately 50% of the efficacy of hCG (FIGURE 14). This analog has also been found to inhibit hCG-induced cyclic-AMP accumulation (FIGURE 14). This reduces the
30 steroidogenic potency of the analog.

It is contemplated that the hCG antagonists would be useful for suppression of gonadal activity during chemotherapy. It is further contemplated that TSH
35 inhibitors would be useful as therapeutics in Graves' disease and that LH inhibitors would be useful for treatment of polycystic ovarian disease.

-23-

OTHER CHIMERAS

5 The invention also relates to other glycoprotein hormone chimeras or analogs. It is contemplated that these chimeras will be useful for structure/function residue mapping, immunological purposes, antagonists, contragestives, and fertility-influencing compounds for various animal species.

10 One important embodiment of the invention is cross species alpha or beta-subunit analogs. Human/bovine alpha-subunit analogs are shown in TABLE IX. These combine with hCG beta-subunit and bind LH receptors (TABLES XI, XIV). It is contemplated that these chimeras
15 will be useful for construction of hormone dimers that can be used as an immunogen such that antibodies cross-reactive with other naturally occurring glycoprotein hormones will not be produced. It is also contemplated that cross-species chimeras may provide alternative
20 sequences to place in human hormones which may further alter the properties of the molecule.

 Another embodiment of the invention demonstrating cross-species subunit chimeras are
25 hCG/bovine LH and hCG/equine LH (or CG) beta-subunit chimeras. Such constructs are shown in TABLE IV and V, respectively. It is contemplated that these constructs will be useful for identifying residues which are important for binding to the human receptor. These may
30 also have applications as immunogens, agonists and/or antagonists.

 Another embodiment of the invention includes hCG/hTSH chimeras. Such constructs are shown in
35 TABLE III. It is contemplated that these analogs will be useful as TSH antagonists and be useful for the treatment of hyperthyroidism.

-24-

It is also within the scope of the invention as illustrated above, that the alpha and beta-subunit sequences in the chimeras can be from any vertebrate (e.g., mammal, bird, amphibian, reptile or fish).

5

Further, the invention contemplates modifying, e.g., increasing the stability of glycoprotein hormones in general, and in particular, of the chimeras of the invention (if desired) as by modifying the orientation and/or number and/or positions of the cysteine residues. It is to be noted that notwithstanding the various structural changes discussed herein, the molecules of the invention fold into the proper conformation as is apparent from the fact that they have biological activity.

15

OTHER ANALOGS

The invention also provides for other glycoprotein hormone analogs wherein methionine and other amino acids have been substituted. Such analogs are shown in TABLES VII and VIII. The structures of the various native glycoprotein hormones are such that they have resisted enzymatic digestion that would permit identification of their disulfide bonds (1, 2). It is contemplated that constructs such as those shown in TABLE VIII, which have been modified to introduce additional sites for cleaving the protein, will be useful for determining the disulfide bonds of the hormones. These particular analogs (containing additional methionine residues) would be cleaved with cyanogen bromide (CNBr) and the sequences of the resulting peptides determined. Based on these sequences, it is possible to identify which Cys residues from particular disulfide-linked pairs. The method can also be used to show that mutagenesis has not altered the "normal" disulfide pattern of analogs such as those described herein, and is another method for examining folding of the protein.

35

METHODS OF USE

5 The compounds of the invention can be
formulated into suitable pharmaceutical, physiologically
active compositions. These compositions may be
administered intravenously, intramuscularly,
parenterally, orally or by other routes appropriate for
proteins. The amount of the active analog of the
10 invention will be used in an amount sufficient to promote
the desired physiological response but preferably not in
an amount in excess of that necessary to cause an optimum
response. The amount may vary from a few nanograms to
about 10 kilograms, the amount being dependent of the
15 human or animal to whom the administration is made, the
condition to be treated and other variables to be taken
into account. One skilled in the art will determine
without undue experimentation the optimum amounts to be
administered. The compositions will normally include a
20 pharmaceutically or physiologically acceptable carrier or
diluent with the active protein of the invention. The
compositions may be in any suitable form, like injectable
preparations.

25 The vaccines of the present invention,
incorporating the compounds of the invention, can be
prepared according to known methods where the antigen is
combined in admixture with a suitable vehicle. Suitable
vehicles include, for example, saline solutions, various
30 known adjuvants, or other additives recognized in the art
for use in vaccine compositions. It is also contemplated
that recombinant cells or organisms, live, attenuated or
dead, might be utilized to deliver antigen(s) for
vaccination. Such vaccines will contain an effective
35 amount of the glycoprotein of the invention and a
suitable amount of vehicle in order to prepare a vaccine
useful for effective administration to the host. For
further background details on antigens, vaccine
development and preparation, see "Antibodies: a

-26-

Laboratory Manual", E. Harlow and D. Lane eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1988; "Advances in Veterinary Science and Comparative Medicine, vol. 33, Vaccine Biotechnology", J.L. Bittle and F.A. Murphy eds., Academic Press, San Diego, 1989 and "New Trends and Developments in Vaccines," A. Volles and H. Friedman, eds., University Park Press, Baltimore, 1978, which are hereby incorporated by reference.

10

EXAMPLES

The following EXAMPLES are provided by way of illustration and not by way of limitation. One skilled in the art is capable without undue experimentation, to vary the components and thus construct other analogs. EXAMPLE 1 describes the insertion of hCG alpha-subunit and beta-subunit cDNAs into the eukaryotic expression vector pSVL. EXAMPLE 2 describes the construction of the vectors pKBM, pKBM-hCG-alpha and pKBM-hCG-beta. EXAMPLE 3 describes the construction of pKBM-hCG-beta', which contains two silent mutations in the hCG-beta cDNA nucleotide sequence resulting in the removal of two restriction sites. EXAMPLE 4 describes the use of cassette mutagenesis to construct analog F8, a deletion construct (see TABLE VII). EXAMPLE 5 describes a chimeric hCG/hFSH beta-subunit, analog B9 (see TABLE II). EXAMPLE 6 describes the construction of a chimeric hCG/hFSH beta-subunit, analog B11 (see TABLE II). EXAMPLE 7 describes the construction of analogs H3 and H6, chimeric human/bovine alpha-subunits (See TABLE IX). EXAMPLE 8 describes the construction of the expression vector pCM, which can be used to stably transfect COS-1 and COS-7 cells. EXAMPLE 9 describes the construction of pBMT2X-hCG-alpha, pBMT2X-hCG-beta and pBMT2X-F8 expression constructs for use in C127 cells. EXAMPLE 10 describes techniques used in mutagenesis and preparation of analogs. EXAMPLE 11 describes transfection of mammalian cells(COS-1, COS-7, C127, CHO) with plasmid DNA. EXAMPLE 12 describes immunological assays of the

. -27-

analogs. EXAMPLE 13 describes assays of receptor binding. EXAMPLE 14 describes assays of receptor stimulation (steroidogenesis or cAMP accumulation).

5 Unless indicated otherwise, standard molecular biological techniques (e.g., ref. 41) were used. Restriction endonucleases and other DNA modification enzymes (e.g., T4 DNA polymerase, *Thermus aquaticus* (Taq) DNA polymerase, T4 DNA ligase, calf intestine alkaline phosphatase, bacterial alkaline phosphatase) were
10 obtained from New England Biolabs, Inc., Bethesda Research Laboratories (Life Technologies), Inc. (BRL), Boehringer Mannheim, United States Biochemical Corp., or Cetus, and were used as suggested by the
15 manufacturer unless indicated otherwise. Subcloning efficiency competent HB101 and DH5-alpha E.coli were obtained from BRL and transformed as suggested by the manufacturer with modifications as noted. DNA sequencing was performed using a Sequenase (TM) kit (United States
20 Biochemical Corp.) as suggested by the manufacturer. COS (COS-1 or COS-7), C 127 and CHO-K1 (CHO) cells were obtained from the American Type Culture Collection (ATCC-Rockville, MD). COS-1, COS-7 and C127 cells were routinely maintained in Dulbecco's modified Eagle's
25 medium (DMEM) containing 10% fetal calf serum (FCS) or 10% supplemented calf serum. CHO cells were cultured in F-12 medium containing 10% FCS as suggested by ATCC. Tissue culture reagents were obtained from GIBCO Laboratories (Life Technologies) Inc. and HyClone
30 Laboratories, Inc. Monoclonal antibodies were provided by Drs. R.E. Canfield (Columbia Univ., New York NY), R.J. Ryan (Mayo Clinic, Rochester, MN), E.G. Armstrong and R. Wolfert (Hybritech Corp., San Diego, CA), and R. Krogsrud and S. Berube (Biomega Diagnostics, Montreal, Canada).

EXAMPLE 1

Construction of pSVL-hCG-beta and pSVL-hCG-alpha

5 The cDNAs for the hCG-alpha and beta-subunits were cloned from human placenta, inserted into the E. coli replicating plasmid pBR322, and sequenced by Fiddes and Goodman (40,43). These clones (pBR322-c-alpha-hCG and pBR322-c-beta-hCG) were obtained from J. Fiddes (California Biotechnology, Mountainview CA) and the cDNA inserts were transferred into the E. coli replicating plasmid/eukaryotic expression vector pSVL (Pharmacia) to create pSVL-hCG-alpha and pSVL-hCG-beta.

15 FIGURE 1 depicts the nucleotide sequence of the hCG beta-subunit cDNA, which includes the coding sequence for the mature protein and signal sequence, as well as adjoining 5' and 3' non-translated regions and a poly(A)/poly(T) region corresponding to the poly(A) tail of the mRNA. The hCG beta-subunit amino acid sequence encoded by the cDNA is depicted using the standard single-letter code (see legend for TABLE I) under the nucleotide sequence. The numbering scheme is the same as that used by Fiddes and Goodman (40). Several restriction sites are shown above the nucleotide sequence. Sites unique in pSVL-hCG-beta or pKBM-hCG-beta (see EXAMPLE 2) are underlined.

30 Ten micrograms (ug) of plasmid pBR322-c-beta-hCG was digested with the restriction enzyme HindIII and the 3'-recessed ends of the cut DNA were filled in with T4 DNA polymerase. The DNA fragment containing the entire 579 base pair (bp) hCG-beta cDNA was purified by electrophoresis in a 1% low melting temperature agarose (Sea Plaque[TM]-FMC, Inc.)/ethidium bromide gel as described elsewhere (42). The DNA fragment containing the hCG-beta cDNA was visualized on a UV light box and recovered by isolating a gel slice containing the DNA with a razor blade. Ten micrograms of plasmid pSVL were

-29-

cut with the restriction enzyme SmaI and the 5'-phosphate groups were removed from the ends of the cut DNA by treatment with calf intestine alkaline phosphatase. The linearized vector DNA (4889 bp) was purified by electrophoresis and isolated as described above. The gel slices containing the hCG-beta cDNA and linearized pSVL vector DNA were melted by heating at 65° C. for 10 minutes, after which 8 microliters of the hCG-beta cDNA were combined with 2 microliters of pSVL and ligated overnight at 15° C. with 400 units T4 DNA ligase (New England Biolabs) in a volume of 20 microliters. The ligation mix was heated at 65° C. for 10 minutes, diluted with 180 microliters of CaCl₂/PIPES buffer (60 mM CaCl₂, 10 mM piperazine-N-N'-bis[2-ethanesulfonic acid], pH 7), and 30 microliters of the diluted reaction was used to transform E. coli DH5-alpha cells. Plasmid DNA from ampicillin-resistant clones were screened by co-digestion with the restriction enzymes PstI and XbaI, which produces a DNA fragment of approximately 233 bp if the cDNA is inserted in the correct orientation (i.e., proper orientation for transcription of sense mRNA).

FIGURE 2 depicts the nucleotide sequence of the hCG alpha-subunit cDNA, which includes the coding sequence for the mature protein and signal sequence, as well as adjoining 5' and 3' non-translated regions. The hCG alpha-subunit amino acid sequence encoded by the cDNA is depicted using the standard single-letter code under the nucleotide sequence. The numbering scheme is the same as that used by Fiddes and Goodman (43). Several restriction sites are shown above the nucleotide sequence. Sites unique in pSVL-hCG-alpha or pKBM-hCG-alpha (see EXAMPLE 2) are underlined. The cDNA for the hCG alpha-subunit (43) was placed into pSVL in the same manner as described for hCG-beta, except that screening for pSVL-hCG-alpha was performed by digestion with XbaI alone and selection of clones exhibiting an approximately 400 bp fragment.

These vectors were used to express hCG subunits together or separately in COS cells (44) and as parent constructs for developing plasmids to express and/or modify glycoprotein hormone subunits. In addition, many of the modifications to the cDNA sequences were made directly in pSVL-hCG-beta and pSVL-hCG-alpha. Other plasmid vectors used in the course of this work are pTZ19R (46), pUCX2 (described below), pBMT2X (47) and pCM (described below).

EXAMPLE 2

Construction of pKBMt, pKBM, pKBM-hCG-alpha, pKBM-hCG-beta, pUCX2

The E. coli replicating plasmid pKBM is a derivative of pUC18 (45) which has been modified to contain the following polylinker, (starting with the HindIII site of pUC18), HindIII, XhoI, XbaI, SacI (SstI), BamHI, BssHII, SalI, SacII (SstII), and EcoRI. This vector was developed to facilitate cassette mutagenesis of glycoprotein hormones. Unlike pSVL, pKBM and pKBMt lack cleavage sites for the restriction enzymes PstI, PpuMI, NaeI and BsmI among others. The absence of these sites in pKBM facilitated cassette mutagenesis using these sites in hCG alpha and beta-subunit cDNAs.

FIGURE 3 depicts the oligodeoxynucleotide cassettes used in the construction of pKBMt (3a) and pKBM (3b). Restriction endonuclease cleavage sites encoded by each cassette are indicated.

Two micrograms of pUC18 were digested with the restriction endonucleases HindIII and XbaI and purified by electrophoresis in low melting point agarose as described in EXAMPLE 1. The two synthetic DNA oligonucleotides depicted in FIGURE 3a were synthesized using an Applied Biosystems 380B DNA Synthesizer and the phosphoramidite method (51), purified by polyacrylamide

gel electrophoresis (as described in Applied Biosystems User Bulletin No. 13, April 1, 1987), and quantified by spectrophotometry (absorbance at 260 nm). The oligonucleotides were then annealed by combining
5 1 microgram of each oligonucleotide in 100 mM KCl, 10 mM Tris pH 8.0, 1 mM EDTA (total volume=200 microliters) heating at 100° C. for five minutes and cooling for 30 minutes at room temperature. Approximately 30 nanograms of HindIII/XbaI cut pUC18 was combined with
10 100 nanograms of the annealed oligonucleotides and ligated overnight at 15° C. with 1 unit T4 DNA ligase (Bethesda Research Laboratories) in a 20 microliter volume. The ligation reaction was heated at 65° C. for 10 minutes and diluted with 180 microliters of
15 CaCl₂/PIPES. Thirty microliters of the diluted ligation reaction was used to transform E. coli K-12 strain JM83 (87) which were then plated with 50 microliters of a 2% 5-bromo,4-chloro,2-indolyl-beta-D-galactoside (X-gal) DMSO solution. Plasmid DNA from ampicillin-resistant
20 blue colonies were screened with XhoI to verify the presence of the cassette. The resulting plasmid was designated pKBMt. To construct the plasmid pKBM, two additional DNA oligonucleotides were synthesized, annealed to form the cassette depicted in FIGURE 3b, and
25 ligated with EcoRI/BamHI digested pKBMt. Plasmid DNA from ampicillin-resistant blue colonies were screened with BssHII, and plasmids containing the new cassette were designated pKBM. The hCG alpha and beta-subunit cDNAs were subsequently transferred from pSVL into pKBM
30 between the XhoI and BamHI sites to create pKBM-hCG-alpha and pKBM-hCG-beta. The vector pUCX2 is another derivative of pUC18 with an altered polylinker. The polylinker in pUCX2 is (starting from the former HindIII site of pUC18), BssHII, SacII, HindIII, XhoI, and EcoRI.

EXAMPLE 3

Construction of pKBM-hCG-beta'

5 An analog of the hCG beta-subunit cDNA, called hCG-beta', containing two silent mutations which eliminate restriction sites has been the starting construct for many mutants.

10 FIGURE 4a depicts the nucleotide sequence of the hCG-beta' DNA. As with the hCG-beta cDNA it includes the coding sequence for the mature protein and signal sequence, adjoining 5' and 3' non-translated regions and a poly(A)/poly(T) region corresponding to the poly(A)
15 tail of the mRNA. The encoded amino acid sequence depicted under the nucleotide sequence is unchanged from that of hCG-beta. The locations of the silent mutations in the DNA sequence are indicated by solid triangles above the base pairs at positions 229 and 232. The
20 numbering scheme is the same as that used by Fiddes and Goodman (40). Several restriction sites are shown above the nucleotide sequence. Sites unique in pSVL-hCG-beta' or pKBM-hCG-beta' are underlined.

25 FIGURE 4b depicts the oligonucleotide cassette used to construct pKBM-hCG-beta'. As in FIGURE 4a, the mutated bases are indicated by solid triangles.

30 Approximately 5 micrograms of pKBM-hCG-beta was digested with the restriction endonucleases PstI and OxaNI (OxaNI is an isoschizomer of Bsu36I and MstII) and the large restriction fragment purified by electrophoresis in low melting point agarose as described above. Two synthetic DNA oligonucleotides (FIGURE 4a)
35 were synthesized, purified and annealed as described in EXAMPLE 2. Two hundred nanograms of PstI/OxaNI cut pKBM-hCG-beta was combined with 80 nanograms of the annealed oligonucleotide cassette, ligated and then used to transform E. coli JM83 as described. Ampicillin-

-33-

resistant white colonies were picked and plasmid DNA from these clones was screened by digestion with the restriction endonuclease PpuMI, which cuts out an approximately 200 bp fragment from pKBM-hCG-beta, but no fragment from pKBM-hCG-beta'. The hCG-beta DNA in pKBM-hCG-beta' differs from the native hCG-beta cDNA in that it contains unique NaeI and PpuMI restriction sites, facilitating its modification by cassette mutagenesis.

10

EXAMPLE 4**Construction of Analog F8**

FIGURE 5a depicts the DNA oligonucleotide cassette used to construct analog F8 and the encoded amino acid sequence. The deletion of residues 47-50 (//) and substitution of proline for the alanine at position 51 (solid triangle) in the native hCG beta-subunit are indicated. These oligonucleotides were synthesized, purified and annealed as described in EXAMPLE 2, and then ligated with PstI/OxaNI digested pKBM-hCG-beta as described in EXAMPLE 3. Plasmid DNA from ampicillin-resistant clones of transformed JM83 E. coli was screened for the presence of the insert by digestion with PpuMI as described in EXAMPLE 2. Plasmids with the proper insert are denoted pKBM-F8 (also referred to as pKBM-beta-delta 3 or pKBM-beta-del 47-50, 51P). The DNA encoding the analog was transferred in to the expression vector pSVL using the restriction endonucleases XhoI and BamHI as described for B9 in EXAMPLE 5, creating pSVL-F8.

30

EXAMPLE 5**Construction of Analog B9**

35

FIGURE 5b depicts the DNA oligonucleotide cassette used to construct analog B9 and the encoded amino acid sequence. The substitution of hFSH beta-subunit residues 88-91 (DSDS) for the corresponding

- 34 -

residues of the hCG beta-subunit, i.e., residues 94-97 (RRST) are indicated by solid triangles, and the recognition site for restriction endonuclease ScaI is also noted. Approximately 25 micrograms of plasmid pKBM-hCG-beta' was used in a time-course digestion with 20 units of restriction endonuclease ApaLI in a 100 microliter volume. A portion of the reaction was removed, placed on ice, and adjusted to 20 mM EDTA at time 0 (12 microliters, taken prior to addition of enzyme), and after 10 (12 microliters), 20 (24 microliters), 30 (24 microliters), 45 (12 microliters) and 60 (16 microliters) minutes incubation at 37° C. These were then purified by electrophoresis in low melting point agarose as described in EXAMPLE 1. In this particular time-course optimal linearization of the plasmid was seen after 20 minutes. The bands containing single cut plasmid (approximately 3280 bp) were cut from the gel, transferred to a microfuge tube, and the gel slices melted by incubating at 65° C. for 10 minutes, after which they were diluted with 200 microliters deionized water. Two hundred microliters of phenol (adjusted as described in ref. 41) were then added, the tube vortexed, and then placed in a dry ice/ethanol bath for 15 minutes, after which they were centrifuged at full speed in an Eppendorf microfuge at -20° C. for 15 minutes. The aqueous fraction was collected and the organic fraction back extracted with an additional 200 microliters deionized water. The aqueous phase from this second extraction was pooled with the aqueous phase from the initial extraction, and then extracted with 400 microliters of chloroform/isoamyl alcohol (24:1 mixture as described in ref. 40), after which the DNA was recovered from the aqueous phase by precipitation with sodium acetate and ethanol. The DNA was resuspended in 14 microliters deionized water, digested with PpuMI, and purified by electrophoresis. The oligonucleotides depicted in FIGURE 5b were synthesized, purified and annealed, and then ligated with the ApaLI/PpuMI digested pKBM-hCG-beta' DNA. Plasmid DNA

- 35 -

from ampicillin-resistant clones of transformed JM83 E. coli was screened for the presence of the insert by digestion with ScaI, which cuts pKBM-hCG-beta' once and cuts pKBM-hCG-beta' containing the insert twice. The new vector is denoted herein as pKBM-B9. The coding sequence for analog B9 was subsequently transferred to pSVL by digesting pKBM-B9 with XhoI and BamHI, gel purifying the approximately 600 bp fragment, and ligating with XhoI/BamHI digested pSVL. Purified preparations of this vector, pSVL-B9, also referred to as pSVL-beta-D or pSVL-beta-CF94-97, were sequenced and used to co-transfect COS7 cells with pSVL-hCG-alpha.

EXAMPLE 6

Construction of Analog B11

In some instances multiple cassettes were ligated into a vector, as for example the assembly of pSVL-B11. This analog, in which amino acids 94-145 in the mature hCG beta-subunit are replaced with amino acids 88-111 from the mature hFSH beta-subunit, was built using plasmid pSVL-B9 (EXAMPLE 6) and two oligonucleotide cassettes.

FIGURE 5c depicts the DNA oligonucleotide cassettes (A and B comprising oligonucleotides A+ and A-, and B+ and B-, respectively) used to construct analog B11 from B9, and the encoded amino acid sequence. The substitution of hFSH beta-subunit residues 95-111 (TVRGLGPSYCSFGEMKE) for the hCG beta-subunit residues 101-145 (GGPKDHPLTCDDPRFQD...Q, see FIGURE 1 for full sequence) are indicated by the solid triangles, and recognition sites for restriction endonucleases StuI and PstI are noted.

The cassettes were designed so that they could be ligated together (they overlap by 4 complementary, non-palindromic bases) and into the ScaI and BamHI sites

of pSVL-B9. The oligonucleotides for the cassettes were synthesized as described for pKBM-hCG-beta' and purified using oligonucleotide purification cartridges from Applied Biosystems. The 5'- ends of oligonucleotides A- and B+ were phosphorylated using T4 polynucleotide kinase to permit ligation of the two cassettes. Oligonucleotides A+ and A-, and B+ and B-, were then combined and annealed as described above. Four micrograms of pSVL-B9 DNA was partially digested with restriction endonuclease ScaI using a time-course, purified by gel electrophoresis, and extracted from the gel as described. The purified ScaI linearized pSVL-B9 DNA was then resuspended in 16 microliters deionized water, digested with restriction endonuclease BamHI, purified by gel electrophoresis, and combined with the two annealed cassettes in a ligation reaction. Ampicillin-resistant clones containing plasmids with the desired insert were isolated by digesting plasmid DNA with PstI, for which there are two recognition sites in pSVL-B9 and three in plasmids with the desired insert, which are denoted pSVL-B11 (also pSVL-beta-DG or pSVL-beta-CF94`117).

EXAMPLE 7

Construction of Analogs H3 and H6

FIGURE 6 depicts the nucleotide sequence of the synthetic bovine alpha-subunit cDNA, constructed from multiple cassettes in pTZ19R, which includes the coding sequence for the mature bovine alpha-subunit protein and the human alpha-subunit signal sequence. The alpha-subunit amino acid sequence encoded by the cDNA is depicted using the standard single-letter code under the nucleotide sequence. Several restriction sites are shown above the nucleotide sequence. Sites unique in pSVL-b-alpha or pTZ19R-b-alpha are underlined. Several restriction sites in common with the native human cDNA

have been used to construct chimeric alpha-subunit analogs.

The XbaI site was used to construct analogs H3 (also referred to as pSVLhum/bov 34) and H6 (also referred to as pSVLbov/hum 34), which are depicted in TABLE IX. To build analog H3, approximately 5 micrograms of pSVL-b-alpha or pSVL-hCG-alpha were digested with the restriction endonucleases XbaI and EcoRV and purified by gel electrophoresis. The large fragment from the digest of pSVL-hCG-alpha and the small fragment from the digest of pSVL-b-alpha were ligated and used to transform DH5-alpha E. coli. Plasmid DNA from ampicillin-resistant clones was screened by digestion with XbaI (pSVL-H3 has single XbaI site, pSVL-hCG-alpha has two). To build analog H6, approximately 2 micrograms of pSVL-b-alpha was digested with restriction endonucleases XbaI and SalI, gel purified, and the large fragment saved. Approximately 5 micrograms of pSVL-hCG-alpha was digested with XbaI and SalI, gel purified and the middle fragment saved. The saved fragments of pSVL-b-alpha and pSVL-hCG-beta were ligated and used to transform DH5-alpha E. coli. Plasmid DNA from ampicillin-resistant clones was screened by digestion with XhoI and BamHI, with positive clones of pSVL-H6 exhibiting an approximately 600 bp fragment as opposed to the approximately 380 bp fragment obtained from pSVL-b-alpha.

EXAMPLE 8

30

Construction of pCM, pCM-hCG-alpha, pCM-hCG-beta

The vector pCM contains the Simian Virus 40 (SV40) origin of replication, early promoter and polyadenylation sequence; the neomycin (aminoglycoside) phosphotransferase gene, ampicillin resistance gene, and bacterial origin of replication (i.e., the EcoRI/BamHI fragment) from pMVBneo (47), the SV40 origin of replication and late promoter, and synthetic polylinker

(XhoI-XbaI-SmaI-SstI-BamHI) from pSVL (i.e., the HindIII/BamHI fragment), and the HindIII/EcoRI fragment of Bovine Papilloma Virus (BPV), which contains BpV genes E6, E7, E8, and a portion of E1, the two plasmid maintenance sequences, and an enhancer region (for review see ref. 48). The HindIII/EcoRI fragment from BPV which we placed into pCM has been shown to be capable of controlling replication of plasmids with an SV40 origin of replication permitting the creation of COS cell lines stably transformed with an episomal vector (49).

The plasmid pCM was constructed as follows. Two micrograms of pSVL was digested with restriction endonucleases EcoRI and BamHI and the smaller fragment (approximately 2000 bp) purified by electrophoresis as described in EXAMPLE 1. Two micrograms of plasmid pMVBneo (47), obtained from G.N.Pavlikis (National Cancer Institute, Frederick, MD), was digested with EcoRI and BamHI and the large fragment (approximately 5000 bp) gel purified. These were then ligated and plasmid DNA from ampicillin-resistant clones of DH5-alpha E. coli screened by digestion with HindIII. Clones exhibiting HindIII cut fragments of approximately 1100, 2950 and 3000 bp were positive for the intermediate plasmid, designated pSVLneo. Additional DNA from these clones was then screened by co-digestion with either EcoRI and BamHI (which was expected to yield fragments of approximately 2000 and 5000 bp) or EcoRI, BamHI and HindIII (which was expected to yield fragments of approximately 450, 500, 1100, 2500 and 2500bp). To assemble the final vector, pCM, approximately 5 micrograms of DNA from a clone of pSVLneo was partially digested with 2 units of HindIII in a reaction from which samples were removed, adjusted to 25 mM EDTA (to inactivate the enzyme) and placed on ice at the following time points; 0 (10 microliters), 10 (22 microliters), 20 (22 microliters), 30 (22 microliters) and 45 (22 microliters) minutes after addition of enzyme. These samples were gel purified and the linearized plasmid (approximately 7000 bp) was cut

-39-

from the gel and transferred to a microfuge tube. The gel slice was melted by incubation at 65° C. for 10 minutes, and 10 microliters was transferred from the microfuge tube to a second tube containing 6 microliters of water, 2 microliters React3 (TM) (BRL) and 20 units of EcoRI. This tube was incubated at 37° C. for an hour, and the approximately 6500 bp EcoRI/HindIII cut band purified by gel electrophoresis. The plasmid pBMT2X (47), obtained from G.N. Pavlakis, was digested with HindIII and EcoRI, gel purified, and the 2200 bp fragment corresponding to the 2200 bp fragment of BPV cut from the gel. This fragment was ligated with the 6500 bp EcoRI/HindIII cut fragment of pSVLneo, and plasmid DNA from ampicillin-resistant clones of DH5-alpha E. coli digested with HindIII to identify plasmids with the 2200 insert from pBMT2X (positive clones exhibit bands of approximately 1100, 3000 and 4800 bp). The new plasmid was designated pCM or pSVLneoBPV. The hCG-beta cDNA was inserted between the XhoI and BamHI sites of pCM and plasmid DNA from ampicillin-resistant clones was screened by digestion with PstI or XhoI and BamHI. This new plasmid was designated pCM-hCG-beta. The hCG-alpha cDNA and DNAs encoding hormone analogs were placed into pCM in similar fashion.

The new vector pCM combines attributes of the two principal existing types of eukaryotic expression systems, i.e., transient high level expression and stable expression, into a single system. When transfected into COS cells using procedures described in EXAMPLE 11, pCM-hCG-beta provided rapid, high level expression of recombinant proteins, superior to that obtained with pSVL-hCG-beta. When these transfected COS cells are incubated with medium containing 500 micrograms/ml of synthetic aminoglycoside G418 (Geneticin-GIBCO) it was possible to establish stably transfected cells which produced recombinant proteins at levels of several hundred nanograms/million cells/day for prolonged periods of time (e.g., >30 cell passages), thereby facilitating

. -40-

the cloning and characterization of gene products. These cells were also successfully frozen, stored in liquid nitrogen for several months, and then recultured without loss of the ability to secrete hCG beta-subunit.

5

It was also possible to recover the DNA vector from the transformed COS cells and reclone it in bacteria. Transformed cells were scraped from the surface of a culture dish and transferred to a 15 ml tube. The cells were then washed twice with 5 ml cold phosphate buffered saline, resuspended in 400 microliters of 0.6% sodium-dodecyl sulfate (SDS)/10 mM EDTA, transferred to a microfuge tube, and incubated for 20 minutes at room temperature. This mixture was then adjusted to 1M NaCl (by addition of 100 microliters of 5M NaCl) and incubated on ice for at least five hours. After this the cellular debris were centrifuged out of solution (full speed in a microfuge for 5 minutes) and the resulting supernatant containing the plasmid was extracted with phenol and precipitated with ethanol and 10 micrograms of mussel glycogen. The isolated DNA was resuspended in 20 microliters of water. One microliter of this was used to transform DH5-alpha E. coli, yielding 63 ampicillin-resistant colonies (we found that these bacteria can also be selected with G418 due to the presence of the neomycin phosphotransferase gene in the vector, even though it is downstream of the SV40 early promoter). Plasmid DNA was prepared from 24 of these clones and digested with XhoI and BamHI. Twenty three of the recovered plasmids were found to contain a fragment of approximately 600 bp corresponding to the hCG-beta cDNA, whereas one clone did not grow in liquid culture. None of the plasmids gave any evidence of rearrangement. This strategy and procedure, previously used with transiently transfected COS cells, is now extended to stable transformed cells containing a hybrid BPV-SV40 vector. Vectors of this type could conceivably be used in any cell type supporting an SV40 origin of replication.

10

15

20

25

30

35

-4/-

Other related viral origins (e.g., polyoma virus) may work in this system as well. Other promoters, enhancers, selectable markers could also be used, singly or in combination. Vectors such as pCM can be used for cloning genes, characterization of gene products, modification of cells, and any other application involving selection of particular transfected cells from a pool of cells. Among the analogs that have been sub-cloned into pCM and expressed in COS cells is analog J2, in which the hCG beta-subunit is anchored to the cell surface via fusion of the transmembrane domain from the Vesicular Stomatitis Virus G-protein (VSV-G) onto the carboxy end of hCG-beta at the SmaI site.

15

TABLE XV illustrates the retention of hCG beta-subunits on COS-7 cells transformed with pCM-beta-J2 and maintained in 500 micrograms/ml G418. COS-7 cells transformed with pCM-hCG-beta do not retain much beta-subunit on their surface. In this assay confluent 75 cm² flasks of cells were cultured for about 30 hours with serum free DMEM containing 500 micrograms/ml G418. The medium was then removed and the cells were gently washed with 10 ml phosphate-buffered saline (PBS). The cells were then removed from the surface of the flask by incubation for 2 hours at 37° C. with PBS containing 0.1% sodium azide/10 mM EDTA. The cells were resuspended in this and 450,000 cells were added to microfuge tubes containing 75,000 cpm ¹²⁵I-labelled antibody B105, and if indicated, 300 nanograms unlabeled B105. These were incubated at 37° C. for an hour, diluted with one ml of a 0.9% saline/0.1% bovine serum albumin solution, and the cells were pelleted by centrifuging for 8 minutes at full speed in a microfuge. The supernatant was aspirated and the pellets counted in a gamma counter.

35

-42-

EXAMPLE 9

Construction of pBMT2X-hCG-alpha, pBMT2X-hCG-beta,
pBMT2X-F8

5 The vector pBMT2X permits stable expression of recombinant proteins in C127 cells (47). hCG subunits and analogs were sub-cloned into the unique XhoI site of pBMT2X (provided by Dr.G.N. Pavlakis, see above, 10 EXAMPLE 8). Approximately 10 micrograms of plasmids pKBM-hCG and pKBM-hCG-beta were digested with restriction endonucleases XhoI and SalI and the approximately 600 bp fragments containing the alpha-subunit and beta-subunit cDNAs were isolated as described 15 in EXAMPLE 1. Two micrograms of plasmid pBMT2X was digested with XhoI, the 5' terminal phosphates removed from the DNA with calf intestinal alkaline phosphatase, and the linearized DNA gel purified as described in example 1. The XhoI digested pBMT2X and cDNA fragments 20 were ligated with T4 DNA ligase and used to transform HB101 E. coli as described for DH5-alpha E. coli in EXAMPLE 1. Plasmid DNA from ampicillin-resistant clones was digested with BssHII, which linearized clones containing the cDNA inserts from pKBM. The orientation 25 of the inserts was determined by digestion of the plasmid DNA with XhoI and SstII, with inserts in the proper orientation exhibiting a fragment of approximately 1400 bp. These new plasmids were designated pBMT2X-hCG-alpha, pBMT2X-hCG-beta. Plasmid preparations of pBMT2X-hCG-alpha and pBMT2X-hCG-beta were subsequently used to 30 transform C127 cells as previously described (50, see also EXAMPLE 11) and cells producing either hCG dimer, or alpha or beta were sub-cloned in the presence of 20-50 uM cadmium chloride (CdCl₂). Production of protein by these 35 cells was on the order of 5 micrograms hCG dimer/million cells/24 hours. DNA encoding analog F8 was subcloned from pKBM-F8 into pBMT2X in similar fashion and subsequently co-transfected with pBMT2X-hCG-alpha into C127 cells and CdCl₂ resistant cells subcloned.

-43-

EXAMPLE 10

METHODS OF MUTAGENESIS

Oligonucleotide Cassette Mutagenesis

5 Many constructs have been prepared by DNA
oligonucleotide cassette mutagenesis similar to the
manner in which pKBMhCG-beta', pKBM-F8, pKBM-B9 or pSVL-
10 B18 were built.

Restriction Fragment Mutagenesis

15 Vectors containing native cDNAs, genes or
analogs thereof with restriction sites in common can be
used to create new analogs by replacing restriction
fragments, as was done in the construction of analogs H3
and H6.

20 Restriction Site Fill-in Insertion Mutagenesis

 The analog G7 (also referred to as hCG-beta-
55iH or hCG-delta 1) was constructed by cutting pSVL-hCG-
beta with OxaNI, filling in the ends of the linearized
25 DNA with T4 DNA Polymerase, religating the vector with T4
DNA ligase, transforming DH5-alpha E.coli, and selecting
clones lacking an OxaNI site. Similar procedures can be
used to mutagenize other restriction sites with a three
base overlap. In addition, cassettes can be used to
30 introduce deletions or insertions.

Site-directed Oligonucleotide Mutagenesis

35 A series of hCG/hLH beta-subunit chimeras were
prepared by oligonucleotide site-directed mutagenesis of
the hCG-beta cDNA. The construction of one of these
constructs, pTZ19R-A4 (also referred to as
pTZ19RhCG/hLH96) is briefly described here. Synthetic
DNA oligonucleotides were prepared which contained

~~44~~

regions of DNA encoding hLH amino acids 96-117 flanked by regions homologous to the hCG-beta cDNA sequence. The hCG-beta cDNA was sub-cloned into pTZ19R between the SacI and SalI sites. The various oligonucleotides were

5 annealed to single stranded vector and used to prime synthesis of double-stranded vector with the Klenow fragment of E. coli polymerase I. The resulting plasmids were used to transform bacteria and colonies containing plasmids with the modified cDNA isolated.

10

Polymerase Chain Reaction Mutagenesis

Several analogs of hCG-beta have been produced using synthetic oligonucleotides and the polymerase chain

15 reaction (PCR). For example, to produce F1, an analog in which the hCG-beta amino acid sequence was truncated at residue 111 (i.e., Cys-110 is the last amino acid) the following oligonucleotide was synthesized, 5'-AAGGACCACCCCTTGACCTGTTAGGATCCTATATA-3', and used as a

20 primer in a PCR reaction with a primer to pSVL which binds 5' to the hCG-beta cDNA. The resulting PCR product was cut with XhoI and BamHI, isolated by gel electrophoresis, and cloned into the XhoI and BamHI sites of pSVL using the techniques described previously.

25

EXAMPLE 11

Introduction of Genetic Vectors into Mammalian Cells

30 Both calcium phosphate and DEAE-dextran methods have been used to transfect COS (COS-1 or COS-7) cells with vectors containing glycoprotein hormone subunits or analogs. The calcium phosphate technique was a modification of a published protocol(50). COS cells to

35 be transfected were plated the day before on 6-cm or 10-cm diameter tissue culture plates at a density of 10,000 cells per square centimeter. Plasmid DNA was purified in an ultracentrifuge over a CsCl gradient and sterilized by precipitation with ethanol. The quantity of DNA used in

-45-

the transfection procedure varied from 1 to 20 micrograms per plate of cells. The final volume of the DNA solution was either 400 microliters (6-cm plate) or 1 ml (10-cm plate). Salmon sperm DNA was sometimes used as a carrier. After resuspending the DNA in sterile water, an appropriate volume of 2 M CaCl_2 was added to yield a final concentration of 125 mM of CaCl_2 in 200 microliters (for each 6-cm plate) or 500 microliters (for each 10-cm plate). An equal volume of 2X BES-buffered saline (50 mM of N-,N-Bis[2-hydroxyethyl]-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na_2HPO_4 ; adjusted to pH 6.95) was then added to the DNA/ CaCl_2 solution. This mixture was vortexed, incubated at room temperature for 10-20 minutes and then added drop-wise to the plates of cells. The cells and DNA were incubated overnight (18-30 hours in a 37° C. incubator under 2.5-6% carbon dioxide. The medium was removed, the cells washed with 1X PBS and then the cells were cultured in complete medium. After 12-24 hours this media was removed, the cells were washed twice with 10 ml PBS, and serum-free DMEM was added. The media was harvested 70-80 hours later for recovery of secreted protein.

Transfections using DEAE-dextran were done according to a published protocol (86). In this procedure, one million cells were plated on 100 mm plates the day before the transfection. Plasmid DNA was prepared and sterilized as described above, then resuspended in sterile water, adjusted to 1x PBS concentration and 200 micrograms/ml DEAE-dextran in 1.2 ml. Media was aspirated from the plates of cells, and the plates were washed twice with PBS. The DNA/DEAE-dextran solution was then added to the plates, which were placed in a 37° C. incubator for 40 minutes. After this time, 10 ml DMEM made 100 μM with chloroquine was added to each plate. The plates were incubated for 3-4 hours, after which the media was replaced with medium containing 10% DMSO. Following 2-3 minutes at 37° C. the medium was aspirated, the plates were washed with PBS, covered with

-46-

10 ml DMEM containing 10% FCS or serum substitute (Calf Supreme or Calf Serum, Supplemented from GIBCO) and placed back in the incubator. After 12-24 hours the medium was removed, the plates washed twice with 10 ml PBS, and then covered with 6 ml serum-free DMEM. The medium was harvested 70-80 hours later for recovery of secreted protein.

Stable transfections of COS, C127 or CHO cells were performed using the calcium phosphate procedure described above. Stable clones of C127 cells transfected with pBMT2X-based plasmids were selected by resistance to CdCl_2 (10-80 mM) starting two days after transfection. Cells transfected with plasmids containing the gene for neomycin (aminoglycoside)phosphotransferase (e.g, pCM-based plasmids) were selected with the synthetic aminoglycoside G418 (Sigma) by adding 500 micrograms/ml media G418/ml tissue culture medium (COS) or 1000 micrograms G418/ml medium(CHO) starting 1-4 days after transfection. After this the cells were routinely maintained under selection pressure.

EXAMPLE 12

Sandwich Assays

Immunological properties of the hormones and analogs were determined using monoclonal sandwich assays essentially as previously described by Moyle et al. (5). These assays were used to determine whether the analogs folded similar to the native molecule and formed alpha, beta-heterodimers which were secreted into the culture medium.

TABLE XIII summarizes results of the immunological characterization of hCG/hFSH chimeras. All assays employed B105 as "capture" antibody, except for assays of hFSH, which used B601, B602, A113 or E501 (see below). The various labeled detection antibodies are

-47-

indicated in the TABLE. The specificity of these antibodies has been described previously (5-7, 52, 53). In these assays, 1 microgram of capture antibody (e.g., B105) was adsorbed to the surface of individual wells in 96-well microtiter plates by adding 50 microliters of a 0.9% NaCl/20 micrograms/ml antibody solution (made with distilled water) to each well and incubating at 37° C. for 1 hour. Unbound antibody was removed and non-specific sites were blocked with bovine serum albumin (BSA) by filling each well with a 0.1% BSA/0.9% NaCl solution and incubating at 37° C. for 1 hour. Hormone standards or analogs were added to each well (volume adjusted to 50 microliters) incubated for 1 hour at 37° C. to permit the analogs to bind to the adsorbed antibody. The wells were washed with distilled water to remove unbound analogs and 50,000 counts per minute (cpm) of labeled "detection" antibody in BSA/saline (50 microliters volume) was added to the well and incubated at 37° C. for 1 hour. Next, the unbound antibody was removed by aspiration, the plates washed with deionized water, and the individual wells cut out and counted in a gamma counter. Detection antibodies were radioiodinated by the iodogen method as previously described by Moyle et al. (54).

25

TABLE XIII illustrates the ability of monoclonal antibody sandwiches which detect the alpha, beta-heterodimer (B105 capture, A113 detection), hCG beta-subunit or hCG (B105 capture, B101 detection), and analogs with beta-subunit epitopes from both hCG (B105) and hFSH (B601). The ability of the radioiodinated detection antibody to bind to the captured hormone or analog relative to hCG (except for B601) is indicated in TABLES XIII. Note that the data in TABLE XIII are expressed as either ++ (cpm bound = 50-120% of those bound to a urinary hCG standard), + (cpm bound = 10-49% of those bound to the standard), or - (<10% binding), and nt indicates not tested. For B601, a monoclonal antibody specific for hFSH, binding to the various analogs is

35

-48-

described relative to their reactivity in an hFSH assay, i.e., E501/B601 sandwich assay. In this assay, employing E501 capture, B601 was found to bind chimeras containing hFSH residues 33-52 indistinguishably from recombinant hFSH. The immunologic data demonstrates that the analogs fold and bind to the alpha-subunit. It also demonstrates that structural features of one hormone (e.g., the B601 epitope in FSH) can be transferred to another hormone, even in regions of very low homology.

10

TABLE XI illustrates monoclonal antibody binding to alpha-subunit analogs co-expressed with hCG beta-subunit. The ability of the radioiodinated detection antibody to bind to the captured hormone or analog relative to hCG is expressed as either ++++(cpm bound = >200% of those bound to a urinary hCG standard), +++(cpm bound = >110% and <200% of those bound to a urinary hCG standard), ++ (cpm bound = 50-110% of those bound to a urinary hCG standard), + (cpm bound = 10-49% of those bound to the standard), or - (<10% binding), and nt indicates not tested. These assays employed B105 as capture antibody. All of these analogs form the alpha, beta-heterodimer (B105 capture, B109 detection). Although antibody B109 is directed against an epitope on the hCG beta-subunit, it binds much better to the alpha, beta-heterodimer than the free beta-subunit, which indicates that it recognizes a conformational change in hCG-beta caused by combination of beta-subunit with alpha-subunit. The data indicate that all of the alpha-subunit analogs are capable of inducing this conformational change in hCG-beta. The data also illustrate that none of the antibodies directed against the hCG-alpha subunit (A101, A102, A109, A112, A202 and A501) bind well to the bovine alpha-subunit. Replacement of bovine amino acid sequence with sequences from the human alpha-subunit enables these antibodies to bind. In some instances, replacement of a discrete contiguous sequence is sufficient to obtain binding similar to that seen with hCG (e.g., A102, A112, A202). For other

-49-

antibodies, a larger contiguous region (A101) or several separate regions (A109, A501) must be substituted to develop antibody binding to the bovine/human chimera. (See TABLE IX for analog sequences) These binding data are summarized in FIGURE 7, which relates binding of hCG alpha-subunit antibodies and the amino acid sequence of the alpha-subunit.

EXAMPLE 13

10

Assay of Receptor Binding

Two types of assays were used to characterize receptor binding. One is a competitive assay while the other is a sandwich or BioIRMA assay. Both have been described previously by Moyle et al. (20, 54).

Membrane bound LH receptors were obtained from rat corpora lutea super-ovulated with pregnant mares serum gonadotropin and hCG (54). Membrane bound FSH receptors were obtained from bovine calf testes (11). In the BioIRMA, hormones or analogs were incubated with a preparation of membrane bound receptors. Hormone which became bound to the receptors was quantified using radiolabeled B105 monoclonal antibody (6) which has high affinity for an epitope on the hCG beta-subunit that remains exposed after the hormone has complexed with receptors.

TABLE XIV illustrates the ability of alpha-subunit analogs co-expressed with hCG beta-subunit to bind LH receptors.

Further characterization of the analog's receptor binding properties was determined by radioligand receptor assays as described by Moyle et al. (20). This assay is a competitive displacement assay in which the analogs compete for receptor binding with radioiodinated hCG and hFSH.

- 52 -

FIGURE 9 illustrates the abilities of hCG, hFSH and analogs to inhibit the binding of ^{125}I -hCG to LH receptors. Their ability to inhibit ^{125}I -hCG binding correlates to their ability to bind LH receptors. Data from each analog are marked using the nomenclature in TABLE II. Data from analogs with hCG-like activity are plotted with filled boxes, rhCG (—), B15 (---), B27 (....); data from analogs with hFSH-like activity are plotted with open boxes, rhFSH (-), B11 (--), B17 (---), B18 (---); and data from analogs that behave differently from both hCG and hFSH are plotted with open triangles, B9 (---) and B16 (---).

FIGURE 10 illustrates the abilities of hCG, hFSH and analogs to inhibit binding of ^{125}I -hFSH to FSH receptors. Their ability to inhibit ^{125}I -hFSH binding correlates to their ability to bind FSH receptors. Representation of hormones and analogs is the same as for LH receptor binding in FIGURE 9.

FIGURE 13 illustrates the ability of the alpha, beta-F8 heterodimer to inhibit ^{125}I -hCG binding to LH receptors. These data show that this analog binds to LH receptors nearly as well as hCG.

For the competitive displacement assays the recombinant hormones and analogs were concentrated using Amicon Centriprep-10 concentrators and quantified by dimer-specific sandwich assays employing monoclonal antibodies to human alpha-subunit (A110, A113) and hCG beta-subunit (B105, B108) using the procedure described above. Urinary derived hCG (CR121 or CR123) was used as the reference standard. Similar results were obtained using either alpha or beta-subunit capture, and with different pairs of antibodies. In addition, analogs that bound anti-hFSH-beta antibody B601 gave comparable results when quantified in an hFSH dimer-specific sandwich assay. Inhibition of labeled hCG binding to LH

-57-

receptors from luteinized rat ovaries was determined as described. The FSH radioligand-receptor assays were conducted essentially as previously described except that the binding incubation was carried out at 37° C. for 2 hours. Bovine testes membranes used in the FSH receptor assays were a gift from Dr.D.J. Bolt (Beltsville Agricultural Research Center, Beltsville, Maryland). Highly purified urinary hCG (CR121 or CR123) and pituitary hFSH (NIADDK-hFSH-I-3 and AFP-4822-B) were used as reference standards and for preparation of label using the iodogen-based procedure. Averaged results from triplicate tubes are presented graphically as percent displacement of total ¹²⁵I-hCG or ¹²⁵I-hFSH binding measured in the absence of inhibitor.

EXAMPLE 14

Assay of Hormone Responses

The ability of hCG, FSH, and the analogs to stimulate steroidogenesis was monitored by radioimmunoassay of testosterone production by rat Leydig cells and estradiol synthesis by rat granulosa cells using procedures similar to those described previously (20, 55).

Rat Leydig cells were isolated and prepared for incubation similar to the procedure described previously (57). Briefly, this procedure consisted of digesting decapsulated rat testes from 22- to 40-day-old Sprague-Dawley rats (Charles River, Wilmington, Mass.), in Krebs Ringer buffer (58) containing 1 mg HEPES in place of bicarbonate and 1 mg collagenase/ml (Worthington, Freehold, N.J.) until the tubules dissociated from one another (10 to 20 minutes). After the addition of 25 ml of enzyme-free buffer to the digestion flasks, the tubules were allowed to sediment and the interstitial cells were decanted into centrifuge tubes. Following centrifugation at 70 x g for 10 minutes the cells were

-52-

resuspended in the buffer containing 1 mg of bovine serum albumin and counted in a hemocytometer. The routine steroidogenesis assay consisted of adding 50 microliters of the cell suspension containing approximately 350,000
5 nucleated cells to 50 microliters of hormone or analog in a plastic tube (12 x 75 mm), and measuring the testosterone produced after a 3-hour incubation at 37° C.

Testosterone was measured with a
10 radioimmunoassay procedure utilizing antisera obtained from Cambridge Medical Diagnostics (Billerica, MA). In this procedure, an aliquot of unextracted incubation medium (50 microliters) containing approximately 175,000 cells was added to 0.2 pmol of ³H-testosterone
15 (80 Ci/mmol, New England Nuclear, Boston, MA) dissolved in 100 microliters of "gelatin/phosphate" buffer. This buffer contained 0.01 M potassium phosphate (pH 7), 0.82% NaCl, 0.1% NaN₃, and 0.2% Difco brand gelatin. Anti-testosterone serum sufficient to bind 0.06 pmol of
20 testosterone was added in 50 microliters of gelatin/phosphate buffer and incubated 1 hour at 37° C. Following an additional 15 minutes in an ice slurry, 400 microliters of dextran/charcoal solution [prepared by adding 1.25 g of powdered charcoal (Sigma, St. Louis, Mo.)
25 to 0.125 g of dextran T-70 (Sigma) and 400 ml of gelatin phosphate buffer] were added to separate the bound and free testosterone. After 15 minutes at 4° C., the mixture was centrifuged at 800 x g for 10 minutes and 400 microliters of the supernatant were added to 4 ml of
30 scintillation fluid (Liquiscent, National Diagnostics). The assay measured 0.02-10 pmol of nonlabeled testosterone.

Granulosa cells were isolated from ovaries of
35 rats (21-27 days old) which had been implanted with 3 diethylstilbestrol (DES) "pellets" made by packing solid DES into 1 cm long sialastic tubings. Estrogen treatment has been shown to induce granulosa cell hyperplasia (60) without altering the number of FSH receptors per cell

(61). Granulosa cells were cultured using a minor modification (59) of the procedure described by Erickson and Hsueh (62). Cells were suspended in McCoy's 5a medium (GIBCO, Grand Island, NY) and either 100 microliters containing 40,000-50,000 cells or one milliliter containing 400,000-800,000 cells was added to each well of a standard 96- or 24-well culture plate. The medium also contained penicillin, streptomycin, and 100 nM androstenedione. Since granulosa cells lack the ability to convert C-21 steroids to C-19 steroids (62), the androstenedione was needed as a substrate for estradiol synthesis. In addition, androstenedione serves as a stimulus for progesterone formation (63, 64) although it is not needed for this function. The medium was not changed during the culture interval. Estradiol and progesterone were measured directly from 0.02-0.2 ml culture fluid by RIA using highly specific antisera (65).

FIGURE 11 is a dose response curve of LH receptor mediated steroidogenic activity of hCG/hFSH chimeric beta-subunits co-expressed in COS7 cells with human alpha-subunit, compared with hCG and hFSH. Data from each analog are marked using the nomenclature from TABLE II. Data from analogs with hCG-like activity are plotted with open boxes, rhCG (—), B15 (— · —), B27 (····); data for rhFSH (—) are plotted with filled boxes; data from analogs with slightly diminished hCG-like activity are plotted with open triangles, B9 (—) and B16 (—); and data from analogs with moderate hCG-like activity are plotted with filled triangles, B11 (—), B17 (—) and B18 (—). The steroidogenic potency of the analogs in this assay is strongly related to their ability their receptor binding activity (see FIGURE 9).

FIGURE 12 is a dose response curve of FSH receptor mediated steroidogenic activity of hCG/hFSH chimeric beta-subunits co-expressed in COS7 cells with human alpha-subunit, compared with hCG and hFSH. Data from each analog are marked using the nomenclature from

TABLE II. Data from analogs with hFSH-like activity are plotted with filled triangles, B11 (--), B17 (---) and B18 (---); data from hFSH (—) are plotted with filled boxes; and data from rhCG (—) are plotted with open boxes. The steroidogenic potency of these analogs in this assay is strongly related to their FSH receptor binding activity (see FIGURE 10).

hCG is known to stimulate adenylyl cyclase activity in testicular interstitial (Leydig) cells. The cAMP which accumulates in response to hormonal stimulation of Leydig cells can be used as a measure of the efficacy of hCG or analogs which interact with LH receptors (20). Leydig cells were prepared by digesting rat testes with collagenase as described above, and 500,000 cells in a volume of 50 microliters were added to assay tubes containing hormone standard or analog to give a final volume of 100 microliters. This mixture was then incubated at 37° C. for 45 minutes, after which the incubation tubes were placed in a boiling waterbath for 5 minutes and then set on ice. Aliquots of the medium or cAMP standards were then incubated with 3H-cAMP and cyclic AMP dependent protein kinase (Sigma, St. Louis) in an ice slurry overnight. Six hundred microliters of dextran-coated charcoal were added to the samples and incubated 10-15 minutes in an ice slurry. The tubes were then centrifuged at 2000 rpm for 15 minutes, and 600 microliters of the supernatant were added to 3 ml of scintillation fluid.

30

FIGURE 14 is a dose response curve of LH receptor mediated cAMP accumulation in response to hCG and in response to F8 which had been co-expressed in C127 cells with alpha-subunit. This FIGURE also indicates the results of incubating hCG and the F8 analog together. FIGURE 14 illustrates that F8 has a reduced efficacy relative to hCG. In addition, F8 inhibits the ability of hCG to stimulate hCG-induced cAMP accumulation.

35

-55-

All references cited hereinabove and hereinafter, are incorporated by reference.

REFERENCES

- 5
1. Pierce, J.G. and Parsons, T.F., Ann. Rev. Biochem. 50, 465 (1981).
 2. Ryan, R.J. et al., Recent Progress Hormone Res. 43, 383 (1987).
 - 10 3. Sairam, M.R., Arch. Biochem. Biophys. 204, 199 (1980).
 4. Keutmann, H.T. et al., Biochem. 22, 3067 (1983).
 5. Moyle, W.R. et al., Proc. Natl. Acad. Sci. (USA) 79, 2245 (1982).
 - 15 6. Ehrlich, P.H. et al., Am. J. Immunol. Microbiol. 8, 48 (1985).
 7. Schwartz, S. et al., Endocrinol. 118, 189 (1986).
 8. Berger, P. et al., Endocrinol. 123, 2351 (1988).
 9. Charlesworth, M.C. et al., J. Biol. Chem. 262, 13409 (1987).
 - 20 10. Keutmann, H.T. et al., Proc. Natl. Acad. Sci. (USA), 84, 2038 (1987).
 11. Sluss, P.M. et al., Biochem. 25, 2644 (1986).
 12. Schneyer, A.L. et al., Biochem. 27, 666 (1988).
 - 25 13. Strickland, T.W. and Puett, D., Int. J. Peptide Protein Res. 21, 374 (1983).
 14. Armstrong, E.G. et al., Biochemical Actions of Hormones (G. Litwack, Ed.), 13, 91 (1986).
 15. Campbell, R.K. et al., Excerpta Medica Int'l. Congr., Series 798, pp. 123-132 (1988).
 - 30 16. Strickland, T.W. and Puett, D., Endocrinol. 111, 95 (1982).
 17. Kusuda, S. and Dufau, M.L., J. Biol. Chem. 261, 16161 (1986).
 - 35 18. Hwang, J. and Menon, K.M.J., Proc. Nat'l. Acad. Sci. (USA) 81, 4667 (1984).
 19. Ji, I. and Ji, T.H., Proc. Nat'l. Acad. Sci. (USA) 78, 5464 (1981).
 20. Moyle, W.R. et al., J. Biol. Chem. 250, 9163 (1975).

- 5 -

21. Goverman, J.M. et al., J. Biol. Chem. 257, 15059 (1982).
22. Matzuk, M.M. et al., 1988 Endocrine Soc. Meeting, New Orleans, Abst. #423 (1988).
- 5 22a. Matzuk, M.M. et al., J. Cell Biol. 109, 1429-1438 (October 1989), The Rockefeller University Press.
23. Calvo, F.O. and Ryan, R.J., Biochem. 24, 1953 (1985).
24. Keutmann, H.T. et al., FEBS Letters 185, 333 (1985).
- 10 25. Bewley, T.A. and Sairam, M.R., Int. J. Peptide Protein Res. 26, 612 (1985).
26. Moyle, W.R. et al., J. Biol. Chem. 262, 16920 (1987).
27. Rebois, R.V. and Fishman, P.H., J. Biol. Chem. 259, 8087 (1984).
- 15 28. Manjunath, P. and Sairam, M.R., J. Biol. Chem. 258, 3553 (1983).
29. Hojo, H. and Ryan, R.J., Endocrinol. 117, 2428 (1985).
- 20 30. Reddy, V.B. et al., Proc. Nat'l. Acad. Sci. (USA) 82, 3644 (1985).
31. Lustbader, J. et al., J. Biol. Chem. 262, 14204 (1987).
32. Corless, C.L. et al., J. Cell Biol. 104, 1173 (1987).
- 25 33. Corless, C.L. et al., J. Biol. Chem. 262, 14197 (1987).
34. Kaetzel, D.M. et al., Proc. Nat'l. Acad. Sci. (USA) 82, 7280 (1985).
- 30 35. Matzuk, M.M. et al., Proc. Nat'l. Acad. Sci. (USA) 84, 6354 (1987).
36. Kaetzel, D.M. and Nilson, J.H., J. Biol. Chem. 263, 6344 (1988).
37. Wang, C., Endocrine Rev. 9, 374 (1988).
- 35 38. Jones, W.R. et al., Lancet 1, 1295 (1988).
39. Gallagher, P. et al., J. Cell. Biol. 107, 2059 (1988).
40. Fiddes, J.C. and Goodman, H.M., Nature 286, 684 (1980).

41. Maniatis, T. et al., "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory (1982).
42. Struhl, K., Biotechniques 3, 6 (1985).
43. Fiddes, J.C. and Goodman, H.M., Nature 281, 351
5 (1979).
44. Gluzman, Y., Cell 23, 175 (1981).
45. Yarnisch-Perron, C. et al., Gene 33, 103 (1985).
46. Mead, D.A. et al., Protein Engineering 1, 67 (1986).
47. Pavlakakis, C.N. et al., "Gene Transfer Vectors for
10 Mammalian Cells", ed. Miller, J.H. and Calos, M.P., Cold
Spring Harbor Laboratory (1987).
48. Stephens, P.E. and Hentschel, C.G., Biochemical J.
248, 1 (1987).
49. Roberts, J.M. and Weintraub, H., Cell 52, 397
15 (1988).
50. Chen, C.A. and Okayama, H., Mol. Cell. Biol. 7, 2745
(1987).
51. Beaucage, S.L. and Caruthers, M.H., Tetrahedron
Letters 22, 1859 (1981).
52. Norman, J. et al., J. Clin. End. Metab. 61, 1031
20 (1985).
53. Milius, R.P. et al., Proc. Nat'l. Acad. Sci. (USA)
80, 7375 (1983).
54. Moyle, W.R. et al., J. Receptor Res. 8, 419 (1988).
55. Skaf, R.A. et al., Endocrinology 117, 106 (1985).
25
56. Dufau, M.L. et al., J. Biol. Chem. 248, 6973 (1973).
57. Moyle, W.R. and Ramachandran, J., Endocrinology 93,
127 (1973).
58. Umbreit, W.W. et al., "Manometric Techniques", 4th
30 ed., page 132, Bruggess Publ. Co. (1964).
59. Moyle, W.R. et al., Am. J. Physiol. 235, E218
(1978).
60. Goldenberg, R.L. and Vaitukaitis, J.L.,
Endocrinology 90, 1492 (1972).
61. Louvet, J.P. and Vaitukaitis, J.L., Endocrinology
35 99, 758 (1976).
62. Erickson, G.F. and Hsueh, A.J.W., Endocrinology 102,
1275 (1978).

63. Nimrod, A. and Lindner, H.R., Mol. Cell Endocrinology 5, 315 (1976).
64. Lucky, A.W. et al., Endocrinology 100, 128 (1977).
65. Powell, J.E. and Stevens, J.C., Clin. Chem. 19, 210 (1973).
- 5 66. Schnever, A.L. et al., Biochemistry 27, 666 (1988).
67. "Recombinant Hormone Challenges Pig Protein for Superovulating Cows", Newswatch, May 4, 1987.
68. Dinsomore, R.P. et al., J. Am. Vet. Med. Assoc. 195, 327 (1989).
- 10 69. Stevens, V.C. et al., Immunology Letters 12, 11 (1986).
70. Sekine, S. et al., Proc. Nat'l. Acad. Sci (USA) 86, 8645 (1989).
- 15 71. Harris, D.C. et al., J. Biol. Chem. 264, 6705 (1989).
72. Lustbader, J.W., et al., Biochem. 28, 9239 (1989).
73. Birken, S. et al., Endocrinology 123, 572 (1988).
74. Krichevsky, A. et al., Endocrinology 123, 584 (1988).
- 20 75. Manning, M.C. et al., Pharm. Res. 6, 903 (1989).
76. Santa Colmoa, T.A. et.al., Biochem. 29, 1194 (1990).
77. Watkins, P.C. et. al., DNA 6, 205 (1987).
78. Noce, T. et al., J. Molec. Endocrinology 3, 129 (1989).
- 25 79. Bousfield, G.R. et al., J. Biol. Chem. 262, 8610 (1987).
80. Talmadge, K., et al., Nature 307, 37 (1984).
81. Maurer, R.A., J. Biol. Chem. 260, 4684 (1985).
- 30 82. Hayashizaki, Y., FEBS Lett. 188, 394 (1985).
83. Virgin, J.B., et al., J. Biol. Chem. 260, 7072 (1985).
84. Goodwin, R.G., et al., Nucleic Acids Res. 11, 6873 (1983).
- 35 85. Stewart, F., et al., J. Endocrinology 115, 341 (1987).
86. Cullen, B. R., Methods Enzymol. 152, 684 (1987).
87. Messing, J., Recombinant DNA Technical Bulletin, NIH, Publication No. 79-009, 2, NO. 2, 43-48, (1979).

-59-

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention and all such modifications are intended to be included within the scope of the following claims.

TABLE I

Examples of Structures of Native Hormones β -subunits

	10	20	30	40	50	60	70	80	90	100	110	120	130	140
HCG	SKEPLRRCRPNATLAVEGCPVCITVNTTICAGCPTMTRV	LGVLPAIPQWCKYRDFESIRLPGCRGVNVSVAVALSCCALCRRSTDCGPKDHPLTCDPRAFQSSSSKAPPSPSRLPGPSDTPILPQ												
HLH	-R-----W-H-----I-----M-----	-A-----P-----T-----D-----FP-----R-GP-----S-----												H-QLSOLLFL
BLH	-RG-----L-Q-----A-----A-----FT-S-----S-K-----	-PVI--PM--R--T--HEL--A--V-----P--D--M--FP-----H-GP--L--S-----												RTQ--A--H-PLP-ILFL
ELH	-RG-----L-----A-----A-----I-----FT-S-----S-V-----	MPAA--I--P--T--EL--A-----P--D--M--FP-----H-GP--QIK-----												VFR-Q--A-APQASS-KDPPSQ-LTST-TPTPGASRRSSHPLPIKTS
CLH	LGGGQ--P-----V-V-----D-----Q-MA-T--A--G-----R-REP-	YRSP-GPP--SA-T-GAL-Y-RMA-W--I--SD-R-LLP-----R--R--PMA-S--TVGGLG-AF-GA-GGFGGE												
SGTI	GTEC-YG--LM-M-I--R-D-HGS--	I-T--L-E-TDLN												
SGTII	S-MQP-Q--Q-VSL-----T-LVIQ-P--S-H-V-KEP-	FKSPFSTVI--H--T-----Y-T-----D--PU-D-H-T-P-----D-S--NMD-S--TIESLQ-DF-ITQ-VLTGDMW												
HFSH	NS-ELT-I-I-I--E-RF--SI--W-----Y-RDL-	YKDPARPKI-KT-TFKELVY-TV-V--AHADSLYT-P--TQ-H-GK-DSDS--TVRGLG-SY-SFGEMKE												
HTSH	F-I-TEY-MHI-RRE-AY-L-I-----M-RDINGKLFIPKY--S-D--T--FIYRTVEI--LH-A-VF--P-----K-GK-MTDYS--IHEAICTNY-TK-QKSYLVGFSV													

alpha-subunits

	10	20	30	40	50	60	70	80	90
human:	APDVVDCEPTLQENPFESQPGAPILQCHGCCSKAYPTPLRSKKTMLVQKNVTSESTCCVAKSYMRVTWGGFKVENHITACHCSTCYHKS								
bovine:	FPDGEFTN-G--K-KY--K-D--Y-----A-----P--I--A-----AFTKA-----NVR-----E-----								
equine:	FPDGEFTT-----K-R--KY--KL-V--Y-----A--R-----P--I-----AFI-----NI-L-----Q-Y-----H--I								
salmon:	YPMSDKTWNG-----KP-TI-PN								

Amino acid sequences of glycoprotein hormone subunits, hCG (40), hLH (80), hLH (81,83), eLH (79), cLH (78), sGTH118 and sGTH118 (77), hFSH (82), human-alpha (43), bovine-alpha (84), equine-alpha (85) and salmon-alpha (70). Single letter amino acid code is used (A=alanine, C=cysteine, D=aspartic acid, E=glutamic acid, F=phenylalanine, G=glycine, H=histidine, I=isoleucine, K=lysine, L=leucine, M=methionine, N=asparagine, P=proline, Q=glutamine, R=arginine, S=s erine, T=threonine, V=valine, W=tryptophan, Y=tyrosine). The β -subunits are from human (h), bovine (b), equine (e), chicken (c) and salmon (s). The sequences are aligned to facilitate examination of homology with either hCG β -subunit (top) or hCG alpha-subunit (bottom). Residues identical to those in the respective hCG subunits are indicated with hyphens (-). The gaps present in the sequences in the figure are due to differences in the number of amino acids in intracysteine regions between TSH and the other β -subunits, and salmon alpha-subunit and the other alpha-subunits. Numbers refer to position in hCG alpha-subunit and β -subunit sequences, other hormone subunits are aligned by their cysteine residues.

TABLE II

Examples of hCG/hFSH CHIMERAS

	10	20	30	40	50	60	70	80	90	100	110	120	130	140
hCG	SKEPLRPRCPINATLAVEKEGCPVCI	TWNTTICAGYCPTRVLGGVL	PALPQWCHYNDVRFESIRLP	GGCGRWNVVSYVALSCCAL	CRRTTDCGPKDHP	LTCDPRFQDSSSSKAPPSLP	SPSRPLGSDTPILLPQ							
hFSH	NS-ELT-I-I-I	---E-RF--SI---	W----	Y-RDL-YKDPARPKI	---KT-TF	KELVY-TV-V	---AHADSLYT-P	---TQ-H-GK-DSDS	---	TVRGLG-SY-SFGEMKE				
B1	NS-ELT-I-I-I	---												
B2	---	E-RF--SI---	W----											
B3	---			Y-RDL										
B4	---				YKDPARPKI									
B5	---					KT-T								
B6	---						FKELVY-TV-V							
B7	---							AHADSLYT-P						
B8	---								TQ-H-GK					
B9	---									DSDS				
B10	---										TVRGLG-SY-SFGEMKE			
B11	---									DSDS		TVRGLG-SY-SFGEMKE		
B12	---											TVRGLG-SY-SFGEMKE		
B13	---									DSDS			TVRGLG-SY-SFGEMKE	
B14	---						Y-RDL	KT-T						
B15	---						Y-RDL-YKDPARPKI	KT-T						
B16	---						Y-RDL-YKDPARPKI	KT-T						
B17	---						Y-RDL-YKDPARPKI	KT-T						
B18	---						Y-RDL-YKDPARPKI	KT-T				TVRGLG-SY-SFGEMKE		
B19	---						Y-RDL-YKDPARPKI	KT-T				TVRGLG-SY-SFGE		
B20	---						Y-RDL-YKDPARPKI	KT-T				TVRGLG-SY-SFGQ		
B21	---										TVRGLG-SY			
B22	NS-ELT-I-I-I	---E-RF--SI---	W----	Y-RDL-YKDPARPKI	KT-TF	KELVY-TV-V	---AHADSLYT-P	---TQ-H-GK						
B23	---											SY-SFGEMKE		
B24	---												GLG-SY-SFGE	
B25	NS-ELT-I-I-I	---E-RF--SI---	W----	Y-RDL-YKDPARPKI	KT-TF	KELVY-TV-V	---AHADSLYT-P	---TQ-H-GK-DSDS						
B26	NS-ELT-I-I-I	---E-RF--SI---	W----	Y-RDL-YKDPARPKI	KT-TF	KELVY-TV-V	---AHADSLYT-P	---TQ-H-GK-DSDS					SY-SFGEMKE	
B27	---													SY-SFGE

See Table I for legend.

TABLE III
Examples of hCG/hTSH CHIMERAS

	10	20	30	40	50	60	70	80	90	100	110	120	130	140
hCG	SKEPLRPRCPINATLAVEKEGCPV	ITVNTTICAGVCP	ITNTRVLQGVLPALPQVVCN	YRDFESTIRLPGCPRGVNPVSV	AVALSCEALCRRSTTDCGPKD	HPLTCD	DRFOOSSSKAPPSLP	SPSR	LPSPSD	PTILPQ				
hTSHB	F-I-TEY-MHI-RRE-AY-L-I	-----	M-RDINGKPKY-S-D-T	-----	FIYRTVEI	-----	LH-A-YF-P	-----	K-GK-NTDYS	-----	IHEAIKTNV-TK-QKSYLVGFSV			
C1	F-I-TEY-MHI	-----												
C2	-----	RRE-AY-L-I	-----											
C3	-----				M-RDINGKPKY-S-D-T	-----								
C4	-----						FIYRTVEI	-----						
C5	-----								LH-A-YF-P	-----				
C6	-----								K-GK-NTDYS	-----				
C7	-----										IHEAIKTNV	-----		
C8	-----												TK-QKSYLVGFSV	
C9	-----											K-GK-NTDYS	-----	IHEAIKTNV-TK-QKSYLVGFSV

-62-

See Table I for legend. In this particular table, "X" denotes the sequence LFL from hTSHB.

TABLE IV
Examples of hCG/BLH CHIMERAS

	10	20	30	40	50	60	70	80	90	100	110	120	130	140
hCG8	SKEPLRPRCPINATLAVEKEGCPVCITWNTTICAGYCPTRVGLPALPQVCHYRDVRFESIRLPGCPGVNPNVYAVALSOCALCRSTTDCGPKDHLTCDPRFQSSSKAPPPSLPSPRLPGSDTPILPQ													
BLH8	-RG---L-Q-----A---A-----FT-S-----S-K---PVI--PM--R--T-HEL--A-V-----P-D-N--FP-----H-GP--L-S-----RTQ--A--H-PLP-ILFL													
D1	-RG---L-Q-----A---A-----													
D2	-----A---A-----FT-S-----													
D3	-----S-K---PVI--PM--R-----													
D4	-----T-HEL--A-V-----													
D5	-----P-D-N--FP-----													
D6	-----H-GP--L-S-----													
D7	-----L-S-----													
D8	-----RTQ--A--H-----													
D9	-RG---L-Q-----A---A-----FT-S-----S-K---PVI--PM--R-----PLP-ILFL													
D10	-RG---L-Q-----A---A-----FT-S-----S-K---PVI--PM--R--T-HEL--A-V-----P-D-N--FP-----H-GP--L-S-----RTQ--A--H-PLP-ILFL													
D11	-----H-GP--L-S-----													
D12	-----RTQ--A--H-PLP-ILFL													

-63-

See Table I for legend.

TABLE V
Examples of hCG/elH CHIMERAS

	10	20	30	40	50	60	70	80	90	100	110	120	130	140
hCG β	SKEPLRPRCRPI	NATLAVEKEGCPV	ITNTTICAGY	CPTMTNRLQGLPALPQ	WVCHYRDRVFESIRLPG	CPRGWIPVWSYAVALSQ	QCALCRSTTDCG	GPKDHP	LTCD	DRFQDSSSK	APPPSLP	SPSR	LPQPSDTP	ILPQ
elH β	RG	L	A	A	A	I	FT	S	S	V	MPAA	I	P	I
E1	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
E2	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
E3	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
E4	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
E5	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

See Table 1 for legend.

TABLE VI
Examples of hCG/hLH CHIMERAS

	10	20	30	40	50	60	70	80	90	100	110	120	130	140
hCG8	SKEPLRPRCP	IMATLAVEKEGCPVCITVNTTICAGYCP	MTNRVLGGVLPALPQVVCNRYRDFESIRLPG	CGPRGVVSVAYALSCQCALCRRSTTCGGPKD	HPLTCD	DPRFQDSSSSKAPPSLP	SPSRLPG	SDT	PI	PQ				
hLH8	-R-----M-H-----I													
A1	-R-----M-H-----I													
A2														
A3														
A3a														
A3b														
A4														
A5														
A6	-R-----M-H-----I													
A7														
A8														
A9	-R-----M-H-----I													
A10														

See Table I for legend.

TABLE VII
Examples of Deletion Mutants

	10	20	30	40	50	60	70	80	90	100	110	120	130	140
hCGB	SKEPLRPRCRP	INATLAVEKEGCPVC	ITVNTTICAGY	QPTNTRVLQGVLP	ALPQVWCKYRDVRF	ESIRLPGCRGVNPNV	SYAVALSCC	ALCRRSTTDCG	GPKOHPLTCDDPR	FGDSSSSK	APPSLPS	PSRLPG	PSDTP	ILPQ
F1	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
F2	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
F3	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
F4	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
F5	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
F6	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
F7	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
F8	-----	-----	-----	-----	P-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

See Table I for legend. Deletions are denoted by empty spaces in place of hCGB residues.

TABLE VIII

Examples of Other Analogs

	10	20	30	40	50	60	70	80	90	100	110	120	130	140
hCG8	SKEPLRPRCPINATLAVEKEGCPVCITVNTTICAGYCPNTNRLQGLPALPQVVCNRYRDRFESIRLPGCPRGVNPVSYAVALSCCALCRRSTTDCGGPKDHPLTCDDPRFQDSSSSKAPPSLPPSRPLGPGSDTPILPQ													
G1	-----H-----													
G2	-----H-----													
G3	-----H-----													
G4	-----H-----													
G5	-----H-----													
G6	-----H-----													
G7	-----H-----													
G8	-----H-----													
G9	-----H-----													
G10	-----H-----													

See Table I for legend. In G9, "X" = any residue except Pro, "J" = Ser or Thr

TABLE IX

Examples of alpha-subunit chimeras and mutants

	10	20	30	40	50	60	70	80	90
human :	APDVDCPECTLQENPFSSPGAP	I	LOCMGCCFSRAYPTPLRSK	TMLVOKNVTSE	STCCVAKSYN	RVTVNGGFKVENHTACH	STCYHKS		
bovine :	FPDGEFTN-G	---K-K-KY--K-D---Y	-----A	-----A	-----P--I--A	-----AFTKA	-----NVR	-----E	---
H1	-----	K-K-KY--K-D---Y	-----A	-----A	-----P--I--A	-----AFTKA	-----NVR	-----E	---
H2	-----	K-K-KY--K-D---Y	-----	-----	-----	-----	-----	-----	---
H3	-----	-----	-----A	-----A	-----P--I--A	-----AFTKA	-----NVR	-----E	---
H4	FPDGEFTN-G	-----	-----	-----	-----	-----	-----	-----	---
H5	FPDGEFTN-G	-----	-----A	-----A	-----P--I--A	-----AFTKA	-----NVR	-----E	---
H6	FPDGEFTN-G	---K-K-KY--K-D---Y	-----	-----	-----	-----	-----	-----	---
H7	FPDGEFTN-G	-----K-D---Y	-----A	-----A	-----P--I--A	-----AFTKA	-----NVR	-----E	---
H8	FPDGEFTN-G	---K-K-KY--K-D---Y	-----A	-----A	-----P--I--A	-----	-----NVR	-----E	---
H9	FPDGEFTN-G	---K-K-KY	-----A	-----A	-----P--I--A	-----AFTKA	-----NVR	-----E	---
H10	FPDGEFTN-G	---K-K-KY--K-D---Y	-----A	-----A	-----P--I--A	-----AFTKA	-----	-----E	---
H11	FPDGEFTN-G	-----K-D---Y	-----	-----	-----	-----	-----	-----	---
H12	FPDGEFTN-G	-----K-D---Y	-----A	-----A	-----P--I--A	-----	-----NVR	-----E	---
H13	FPDGEFTN-G	-----	-----A	-----A	-----P--I--A	-----	-----NVR	-----E	---
H14	-----	-----	-----	-----	-----	-----	-----	-----	---
H15	-----	-----	-----	-----	-----	-----	-----M	-----	---
H16	-----	-----	-----	-----	-----	-----	-----	-----N--I	---
H17	-----	-----	-----	-----	-----	-----	-----	-----HHH	---
H18	-----	-----	-----	-----	-----	-----	-----	-----	---
H19	FPDGEFTN-G	-----	-----	-----	-----	-----	-----	-----	---
							---AFTKA	---NVR	---

See Table I for legend. The gap in H19 is a deletion mutation.

TABLE X
Examples of Fusion Constructs

	10	20	30	40	50	60	70	80	90	100	110	120	130	140
hCG8	SKEPLRRCRP	INATLAVEKEGCP	VCITVMITICAGYCP	TRVLGVL	PALPOVWCVNRD	VRFE	SIRLPGCP	GNPVSYA	VALSCCAL	CRRTDCGG	KDHP	LTCDPR	FDSSSKAPP	SLPSPRLPGPSDTP
J1	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	----- (hCG-alpha)
J2	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	----- (VSV-G) ²
J3	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	----- (IgG) ³
J4	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	----- (CAT) ⁴
J5	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	----- (Strep) ⁵
J6	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	----- [second protein]
J7	DD [Z]													

-69-

See Table 1 for legend.

- 1 Fusion protein with entire hCG alpha-subunit amino acid residues -24 to +92 (see Figure 2).
- 2 Fusion protein with transmembrane and cytoplasmic domains of vesicular stomatitis virus (VSV) G- protein.
- 3 Fusion protein with hCG8 in place of immunoglobulin variable region.
- 4 Fusion protein with chloramphenicol acetyltransferase.
- 5 Fusion protein with streptavidin.
- 6 Fusion protein with hCG8 residues 9-110 plus any of the proteins in J1-5.
- 7 Fusion protein with any glycoprotein hormone 8-subunit [X] plus a second protein [Z] such as an enzyme or binding protein.

-70-

TABLE XI
MONOCLONAL ANTIBODY BINDING TO HUMAN/BOVINE alpha-SUBUNIT
CHIMERAS CO-EXPRESSED WITH hCG 8-SUBUNIT

alpha-subunit or Analog	Monoclonal Antibody					
	B109	A101	A102	A109	A112	A202 A501
uhCG	++	++	++	++	++	++
rhCG	++	++	++	++	++	++
b/+hCG8	++	-	-	-	-	-
H1	++	-	-	-	-	-
H2	++	-	-	-	++	++
H3	++	++	++	+	-	+
H4	++	++	++	++	++	++
H5	++	++	++	+	-	+
H6	++	-	-	-	++	++
H7	++	+	++	-	-	-
H8	++	-	-	-	++	++
H11	++	nt	++	++	nt	++
H12	++	nt	++	+	nt	++
H14	++	++	++	+++	++	+++

Data are presented as binding of labeled antibody to chimeric proteins relative to binding to urinary hCG; +++ = greater than 200% cpm bound by hCG standard, ++ = greater than 110%, + = 50%-110%, - = less than 10% and nt = not tested.

-71-

TABLE XII

Monoclonal Antibody Binding to Membranes from COS7 Cells
Expressing hCG/VSF-G Fusion Proteins +/- β -Subunit

Transfection		Ratio MAb/MAb+hCG	
α hCG+ β tail ¹	β tail ¹	MAb	α hCG+ β ¹
A111	6954	952	
A111 + 1 / α hCG	1024	1178	A111 6.8 0.8
B109	6056	1998	
B109 + 1 / α hCG	1346	1514	B109 4.5 1.3
B105	11872	26104	
B105 + 1 / α hCG	1049	1268	B105 11.3 20.6

1. β analog J2 (hCG/VSF-G fusion protein)

-72-

TABLE XIII
MONOCLONAL ANTIBODY BINDING TO CHIMERIC hCG/hFSH
B-SUBUNITS CO-EXPRESSED WITH alpha-SUBUNIT

B-Subunit or Analog	A113	B101	B109	B601	A109
rhCG	++	++	++	-	++
B3	++	nt	++	nt	+
B9	++	++	-	-	++
B11	++	++	nt	-	nt
B14	++	nt	-	nt	+
B15	++	-	-	++	-
B16	++	-	-	++	-
B17	++	-	nt	++	nt
B18	++	++	-	-	++
B28	++	-	-	++	-
B27	++	++	nt	-	nt
rhFSH	++	-	-	++	nt

Data are presented as antibody binding to chimeric proteins relative to binding to hCG or hFSH standard; ++ = 50%-110%, + = 10%-50%, - = less than 10%, nt = not tested

-73-

TABLE XIV
 BINDING OF HUMAN/BOVINE α -SUBUNIT CHIMERAS CO-EXPRESSED
 WITH hCG β -SUBUNIT TO LH RECEPTORS

α -Subunit BioIRMA	
or Analog	B105
uhCG	++
rhCG	++
b/hCG8	++
H1	++
H2	++
H3	++
H4	++
H5	++
H6	++
H7	++
H8	++

Data are presented as binding of mAb B105 to hormone analog/receptor complex relative to binding to hCG/receptor complex; ++ = 50%-150%, + = 10%-49%, = less than 10%

-74-

TABLE XV
MONOCLONAL ANTIBODY BINDING TO STABLY TRANSFECTED COS7 CELLS
EXPRESSING hCG B-SUBUNIT OR hCG8/VSV-G

Cell	B105	B105+300ng hCG	Ratio +/- hCG
COS7-hCG8	4772	2244	2.1
COS7-hCG8/VSV-G (J2)	45762	1887	24.3

We claim:

-75-

1. A physiologically active glycoprotein hormone analog which has coupled alpha and beta-subunits which glycoprotein has bonding affinity to two receptors of different native glycoprotein hormones, with the proviso that the analog is not human LH-beta-CG-beta wherein the beta-subunit binds to a common gonadal receptor.

10

2. A physiologically active glycoprotein hormone analog which has coupled alpha and beta-subunits which has binding affinity to two different receptors.

15

3. A physiologically active glycoprotein hormone analog which has coupled alpha and beta-subunits which has binding affinity to two receptors of different native glycoprotein hormones with the proviso that either one of the subunits is a species other than human.

20

4. The physiologically active glycoprotein hormone analog according to claim 2, wherein the beta-subunit contains amino acid sequences found in native CG and FSH.

25

5. The physiologically active glycoprotein hormone analog according to claim 2, wherein the beta-subunit contains amino acid sequences found in native CG and TSH.

30

6. The physiologically active glycoprotein hormone analog according to claim 4, wherein at least one of the subunits is human.

35

7. The physiologically active glycoprotein hormone analog according to claim 5, wherein at least one of the subunits is human.

-76-

8. The physiologically active glycoprotein hormone analog according to claim 6, wherein the beta-subunit is human.

5 9. The physiologically active glycoprotein hormone analog according to claim 7, wherein the beta-subunit is human.

10 10. The physiologically active glycoprotein hormone analog according to claim 3, wherein the subunit is other than human and is selected from the group consisting of bovine, equine, ovine, and pisces.

15 11. The physiologically active glycoprotein hormone analog according to claim 10, wherein the beta-subunit is other than human.

Figure 1. hCG B-subunit cDNA and protein sequence

1	10	20	30	40	50	60
5'-AGACAAGGCAGGGGACGCACCAAGG ATG GAG ATG TTC CAG GGG CTG CTG CTG TTG CTG CTG CGT AGC-3'						
3'-TCTGTTCGGTCCCTGCGTG GTTC TAC CTC TAC AAG GTC CCC GAC GAC GAC AAC GAC GAC GAC TCG-5'						
M E M F Q G L L L L L L S						
			-20			
-10						
70	80	90	100	110	120	130
5'-ATG GGC GGG ACA TGG GCA TCC AAG GAG CCG CTT CGG CCA CGG TGC CGC CCC ATC AAT GCC ACC-3'						
3'-TAC CCG CCC TGT ACC CGT AGG TTC CTC GGC GAA GCC GGT GCC ACG GCG GGG TAG TTA CGG TGG-5'						
M G G T W A S K E P L R P R C R P I N A T						
			-1	1	10	
<u>HincII</u> <u>NaeI</u>						
140	150	160	170	180	190	
5'-CTG GCT GTG GAG AAG GAG GGC TGC CCC GTG TGC ATC ACC GTC AAC ACC ACC ATC TGT GCC GGC-3'						
3'-GAC CGA CAC CTC TTC CTC CCG ACG GGG CAC ACG TAG TGG CAG TTG TGG TGG TAG ACA CGG CCG-5'						
L A V E K E G C P V C I T V N T T I C A G						
20			30			
<u>PstI</u> <u>PpuMI</u> <u>NaeI</u> <u>Bsu36I</u>						
200	210	220	230	240	250	
5'-TAC TGC CCC ACC ATG ACC CGC GTG CTG CAG GGG GTC CTG CCG GCC CTG CCT CAG GTG GTG TGC-3'						
3'-ATG ACG GGG TGG TAC TGG GCG CAC GAC GTC CCC CAG GAC GGC CGG GAC GGA GTC CAC CAC ACG-5'						
Y C P T M T R V L Q G V L P A L P Q V V C						
40			50			

Figure 1. hCG B-subunit cDNA and protein sequence (continued)

TaqI

260	270	280	290	300	310
5'-AAC TAC CGC GAT GTG CGC TTC GAG TCC ATC CGG CTC CCT GGC TGC CCG CGC GGC GTG AAC CCC-3'					
3'-TTG ATG GCG CTA CAC GCG AAG CTC AGG TAG GCC GAG GGA CCG ACG GGC GCG CCG CAC TTG GGG-5'					
N Y R D V R F E S I R L P G C P R G V N P					
60			70		

Pvu II Apa LI

320	330	340	350	360	370	380
5'-GTG GTC TCC TAC GCC GTG GCT CTC AGC TGT CAA TGT GCA CTC TGC CGC CGC AGC ACC ACT GAC-3'						
3'-CAC CAG AGG ATG CCG CAC CGA GAG TCG ACA GTT ACA CGT GAG ACG GCG GCG TCG TGG TGA CTG-5'						
V V S Y A V A L S C Q C A L C R R S T T D						
80			90			

PpuMI

390	400	410	420	430	440
5'-TGC GGG GGT CCC AAG GAC CAC CCC TTG ACC TGT GAT GAC CCC CGC TTC CAG GAC TCC TCT TCC-3'					
3'-ACG CCC CCA GGG TTC CTG GTG GGG AAC TGG ACA CTA CTG GGG GCG AAG GTC CTG AGG AGA AGG-5'					
C G G P K D H P L T C D D P R F Q D S S S					
100		110		120	

ApaI

SmaI

450	460	470	480	490	500
5'-TCA AAG GCC CCT CCC CCC AGC CTT CCA AGC CCA TCC CGA CTC CCG GGG CCC TCG GAC ACC CCG-3'					
3'-AGT TTC CCG GGA GGG GGG TCG GAA GGT TCG GGT AGG GCT GAG GGC CCC GGG AGC CTC TGG GGC-5'					
S K A P P P S L P S P S R L P G P S D T P					
130				140	

510 520 530 539

5'-ATC CTC CCA CAA TAA AGGCTTCTCAATCCGC[A]₄₀-3'

3'-TAG GAG GGT GTT ATT TCCGAAGAGTTAGGCG[T]₄₀-5'

I L P Q ***

3/16

Figure 2. hCG alpha-subunit cDNA and protein sequence

1 50
HincII NcoI
 5'-CAGTCAACCGCCCTGAACACATCCTGCAAAAAGCCAGAGAAAGGAGCGCC ATG GAT TAC TAC AGA AAA TAT-3'
 3'-GTCAGTTGGCGGGACTTGTGTAGGACGTTTTTCGGGTCTCTTTCCTCGCGG TAC CTA ATG ATG TCT TTT ATA-5'
 M D Y Y R K Y
 -24 -20

100
 5'-GCA GCT ATC TTT CTG GTC ACA TTG TCG GTG TTT CTG CAT GTT CTC CAT TCC GCT CCT GAT-3'
 3'-CGT CGA TAG AAA GAC CAG TGT AAC AGC CAC AAA GAC GTA CAA GAG GTA AGG CGA GGA CTA-5'
 A A I F L V T L S V F L H V L H S A P D
 -10 -1 1

150
BsmI
 5'-GTG CAG GAT TGC CCA GAA TGC ACG CTA CAG GAA AAC CCA TTC TTC TCC CAG CCG GGT GCC-3'
 3'-CAC GTC CTA ACG GGT CTT ACG TGC GAT GTC CTT TTG GGT AAG AAG AGG GTC GGC CCA CGG-5'
 V Q D C P E C T L Q E N P F F S Q P G A
 10 20

200 250
XbaI
 5'-CCA ATA CTT CAG TGC ATG GGC TGC TGC TTC TCT AGA GCA TAT CCC ACT CCA CTA AGG TCC-3'
 3'-GGT TAT GAA GTC ACG TAC CCG ACG ACG AAG AGA TCT CGT ATA GGG TGA GGT GAT TCC AGG-5'
 P I L Q C M G C C F S R A Y P T P L R S
 30 40

300
 5'-AAG AAG ACG ATG TTG GTC CAA AAG AAC GTC ACC TCA GAG TCC ACT TGC TGT GTA GCT AAA-3'
 3'-TTC TTC TGC TAC AAC CAG GTT TTC TTG CAG TGG AGT CTC AGG TGA ACG ACA CAT CGA TTT-5'
 K K T M L V Q K N V T S E S T C C V A K
 50 60

350 400
DraIII PstI
 5'-TCA TAT AAC AGG GTC ACA GTA ATG GGG GGT TTC AAA GTG GAG AAC CAC ACG GCG TGC CAC-3'
 3'-AGT ATA TTG TCC CAG TGT CAT TAC CCC CCA AAG TTT CAC CTC TTG GTG TGC CGC ACG GTG-5'
 S Y N R V T V M G G F K V E N H T A C H
 70 80

450 500
ScaI
 5'-TGC AGT ACT TGT TAT TAT CAC AAA TCT TAA ATGTTTACCAAGTGCTGTCTTGATGACTGCTGATTTTC-3'
 3'-ACG TCA TGA ACA ATA ATA GTG TTT AGA ATT TACAAATGGTTCACTACAGAACTACTGACGACTAAAAG-5'
 C S T C Y Y H K S ***
 90 92

550
PstI
 5'-ACGCTTCAAGGATATACGACGCTTACTGCCTTCCTCCTTATCCTACAGTACAATCAGCAGTCTAGTCTTTTCATTT-3'
 3'-TGCGAAGTTCCTATATGACGTCGAAATGACGGAAGGAGGAATAGGATGTCATGTTAGTCGTGATCAAGAAAAGTAAA-5'

600
HindIII
 5'-GGAATGAATACAGCATTAAAGCTT-3'
 3'-CCTTACTTATGTCGTAATTCGAA-5'

4/16
FIGURE 3. Oligonucleotides used to construct pKBM and pKBmt

3a pUC18 / pKBmt

HindIII XbaI

XhoI

5'-AGCTTCTCGAGT-3'

3'-AGAGCTCAGATC-5'

3b pKBmt / pKBM

BamHI

SalI

EcoRI

BssHII

SacII

5'-GATCCGCGCGCGTCGACCCGCGGG-3'

3'-GCGCGCGCAGCTGGGCGCCCTTAA-5'

Figure 4a. hCGB' DNA and protein sequence

1 10 20 30 40 50 60

5'-AGACAAGGCAGGGGACGCACCAAGG ATG GAG ATG TTC CAG GGG CTG CTG CTG TTG CTG CTG CGT AGC-3'

3'-TCTGTTCCGTCCCCTGCGTGGT7CC TAC CTC TAC AAG GTC CCC GAC GAC GAC AAC GAC GAC GAC TCG-5'

M E M F Q G L L L L L L L S

-20 -10

70 80 90 100 110 120 130

5'-ATG GGC GGG ACA TGG GCA TCC AAG GAG CCG CTT CGG CCA CGG TGC CGC CCC ATC AAT GCC ACC-3'

3'-TAC CCG CCC TGT ACC CGT AGG TTC CTC GGC GAA GCC GGT GCC ACG GCG GGG TAG TTA CGG TGG-5'

M G G T W A S K E P L R P R C R P I N A T

-1 1 10

140 150 160 170 180 190

5'-CTG GCT GTG GAG AAG GAG GGC TGC CCC GTG TGC ATC ACC GTC AAC ACC ACC ATC TGT GCC GGC-3'

3'-GAC CGA CAC CTC TTC CTC CCG ACG GGG CAC ACG TAG TGG CAG TTG TGG TGG TAG ACA CGG CCG-5'

L A V E K E G C P V C I T V N T T I C A G

20 30

HincII Nae I

200 210 220 230 240 250

5'-TAC TGC CCC ACC ATG ACC CGC GTG CTG CAG GGG GTG CTC CCG GCC CTG CCT CAG GTG GTG TGC-3'

3'-ATG ACG GGG TGG TAC TGG GCG CAC GAC GTC CCC CAC GAG GGC CGG GAC GGA GTC CAC CAC ACG-5'

Y C P T M T R V L Q G V L P A L P Q V V C

40 50

PstI / / Bsu36I

260 270 280 290 300 310

5'-AAC TAC CGC GAT GTG CGC TTC GAG TCC ATC CGG CTC CCT GGC TGC CCG CGC GGC GTG AAC CCC-3'

3'-TTG ATG GCG CTA CAC GCG AAG CTC ACG TAG GCC GAG GGA CCG ACG GGC GCG CCG CAC TTG GGG-5'

N Y R D V R F E S I R L P G C P R G V N P

60 70

TaqI

320 330 340 350 360 370 380

5'-GTG GTC TCC TAC GCC GTG GCT CTC AGC TGT CAA TGT GCA CTC TGC CGC CGC AGC ACC ACT GAC-3'

3'-CAC CAG AGG ATG CGG CAC CGA GAG TCG ACA GTT ACA CGT GAG ACG GCG GCG TCG TGG TGA CTG-5'

V V S Y A V A L S C Q C A L C R R S T T D

80 90

Pvu II Apa LI

390 400 410 420 430 440

5'-TGC GGG GGT CCC AAG GAC CAC CCC TTG ACC TGT GAT GAC CCC CGC TTC CAG GAC TCC TCT TCC-3'

3'-ACG CCC CCA GGG TTC CTG GTG GGG AAC TGG ACA CTA CTG GGG GCG AAG GTC CTG AGG AGA AGG-5'

C G G P K D H P L T C D D P R F Q D S S S

100 110 120

PpuMI

450 460 470 480 490 500

5'-TCA AAG GCC CCT CCC CCC AGC CTT CCA AGC CCA TCC CGA CTC CCG GGG CCC TCG GAC ACC CCG-3'

3'-AGT TTC CGG GGA GGG GGG TCG GAA GGT TCG GGT AGG GCT GAG GGC CCC GGG AGC CTC TGG GGC-5'

S K A P P P S L P S P S R L P G P S D T P

130 140

SmaI ApaI

510 520 530 539

5'-ATC CTC CCA CAA TAA AGGCTTCTCAATCCGC(A) 40-3'

3'-TAG GAG GGT GTT ATT TCCGAAGAGTTAGGCG(T) 40-5'

I L P Q ***

6/16

Figure 4b. Oligonucleotides used to construct hCG8'

pKBM-hCG8 - pKBM-hCG8'

PstI

v v

OxaNI(Bsu361)

5'-G GGG GTG CTC CCG GCC CTG CC-3'

3'-AC GTC CCC CAC GAG GGC CCG GAC GGA GT-5'

L Q G V L P A L P Q

7/16

Figure 5. Oligonucleotides used to construct analogs F8, B9 and B11

5a Analog F8

PstI OxaNI(Bsu361)

5'-G CCC CTG CC-3'

3'-AC GTC GGG GAC GGA GT-5'

L Q P L P Q

45 46/51 52 53 54

5b Analog B9

ApaLI ScaI PpuMI

5'-T GCA CTC TGC GAC AGC GAC AGT ACT GAC TGC GGG G-3'

3'-GAG ACG CTG TCG CTG TCA TGA CTG ACG CCC CCA G-5'

C A L C D S D S T D C G G P

90 94 95 96 97 100

5c Analog B11;

ScaI StuI

Cassette A, A+ 5'-ACT GAC TGC ACC GTG AGA GGC CTC GGA CC-3'

A- 3'-TGA CTG ACG TGG CAC TCT CCG GAG CCT GGG AGA-5'

T D C T V R G L G P S

100 ^ ^ ^ ^ ^ ^ ^

PstI BamHI

Cassette B, B+ 5'-C TCT TAC TGC AGC TTT GGT GAA ATG AAA GAA TAA G-3'

B- 3'-ATG ACG TCG AAA CCA CTT TAC TTT CTT ATT CCTAGG-5'

Y C S F G E M K E ***

^ 110 ^ ^ ^ ^ ^ ^ ^

8/16

Figure 6. Synthetic bovine alpha-subunit DNA and protein sequence

EcoRI /XhoI

1 10 20 30 40 50

5'-GAATTCCTCGAGGGG ATG GAT TAC TAC AGA AAA TAT GCT GCC ATC TTT CTG GTG-3'

3'-CTTAAGGAGCTCCCC TAC CTA ATG ATG TCT TTT ATA CGA CGG TAG AAA GAC CAC-5'

M D Y Y R K Y A A I F L V

-24 -20

DraIII BspMI

60 70 80 90 100

5'-ACA CTG AGT GTG TTT CTG CAT GTT CTC CAT TCC TTT CCG GAT GGA GAG TTT-3'

3'-TGT GAC TCA CAC AAA GAC GTA CAA GAG GTA ACG AAA GGC CTA CCT CTC AAA-5'

T L S V F L H V L H S F P D G E F

-10 -1 1

BsmI FspII

110 120 130 140 150

5'-ACA ATG CAA GGC TGT CCT GAA TGC AAG CTA AAA GAA AAC AAA TAC TTT TCG-3'

3'-TGT TAC GTT CCG ACA GGA CTT ACG TTC GAT TTT CTT TTG TTT ATG AAA AGC-5'

T M Q G C P E C K L K E N K Y F S

10 20

NsiI XbaI

160 170 180 190 200

5'-AAG CCA GAT GCT CCA ATC TAT CAA TGC ATG GGG TGC TGC TTC TCT AGA GCA-3'

3'-TTC GGT CTA CGA GGT TAG ATA GTT ACG TAC CCC ACG ACG AAG AGA TCT CGT-5'

K P D A P I Y Q C M G C C F S R A

30 40

BglII

210 220 230 240 250

5'-TAC CCC ACT CCA GCG AGA TCT AAG AAG ACA ATG TTG GTC CCC AAG AAC ATC-3'

3'-ATG GGG TGA GGT CGC TCT AGA TTC TTC TGT TAC AAC CAG GGG TTC TTG TAG-5'

Y P T P A R S K K T M L V P K N I

50

SpeI BclI BstXI

260 270 280 290 300

5'-ACT AGT GAA GCT ACA TGC TGT GTG GCC AAA GCA TTT ACC AAG GCC ACA GTG-3'

3'-TGA TCA CTT CGA TGT ACG ACA CAC CGG TTT CGT AAA TGG TTC CGG TGT CAC-5'

T S E A T C C V A K A F T K A T V

60 70

Scal

DraIII PstI

310 320 330 340 350 360

5'-ATG GGA AAT GTC AGA GTG GAG AAC CAC ACC GAG TGC CAC TGC AGT ACT TGT-3'

3'-TAC CCT TTA CAG TCT CAC CTC TTG GTG TGG CTC ACG GTG ACG TCA TGA ACA-5'

M G N V R V E N H T E C H C S T C

80 90

SacII/BamHI/HindIII

370 380 396

5'-TAT TAT CAC AAA TCT TAA CCGCGGGGATCCAAGCTT

3'-ATA ATA GTG TTT AGA ATT GCGCCCTAGGTTGAA

Y Y H K S ***

9/16

Figure 7. Regions of human alpha-subunit involved in antibody binding

	10	20	30	40	50	60	70	80	90
	A101,A109,A501								
	/-----/					A202,A112			
	A102					A109,A501			
	/-----/					/---/			
human :	APDVQDCPECTLQENPFFSQPGAPILQCMGCCFSRAYPTPLRSKKTMLVQKNVTSESTCCVAKSYNHRVTVMGGFKVENHTACHCSTCYHK								
bovine:	FPDGEFTM-G---K-K--KY--K-D---Y-----A-----P--I---A-----AFTKA---NVR-----E-----								

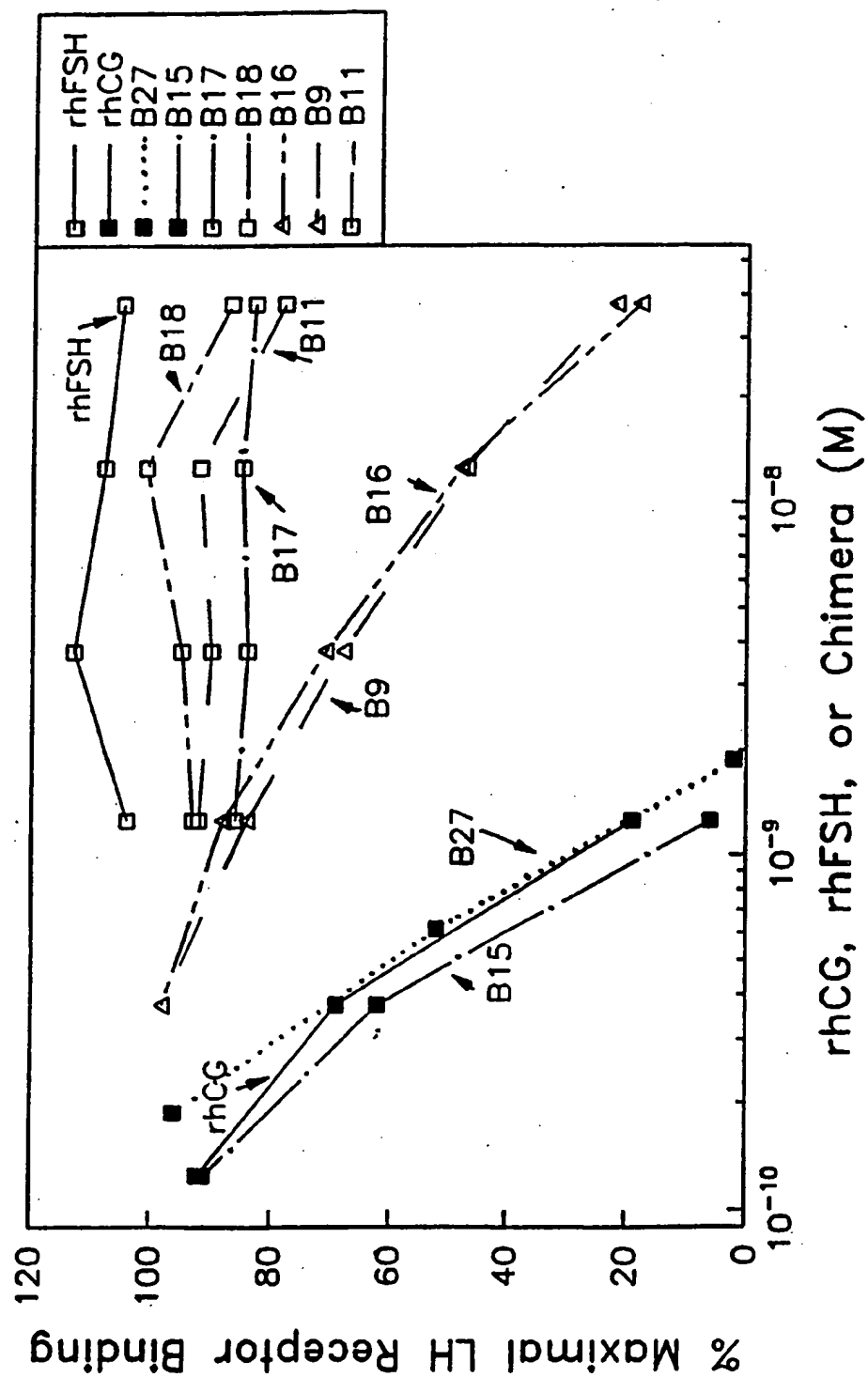
10/16

FIGURE 8

BOG AND HLE RESIDUES WHICH DETERMINE ANTIBODY BINDING SPECIFICITY

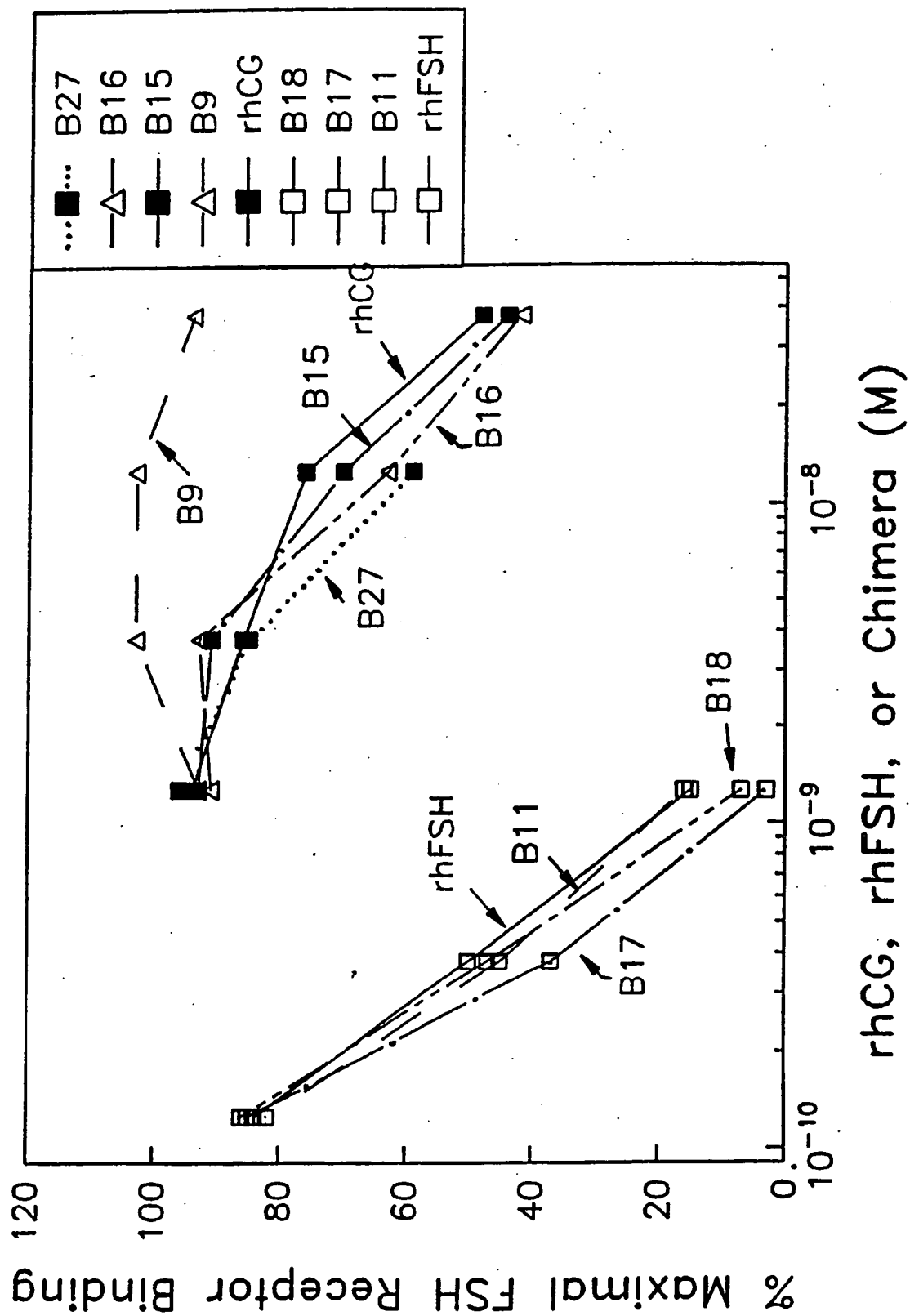
		<u>B204</u>	20
hCG	SerLysGluProLeuArgProArgCysArgProIleAsnAlaThrLeuAlaValGluLys		
hLH	- Arg - - - - Trp - His - - - Ile - - - -		
			40
hCG	GluGlyCysProValCysIleThrValAsnThrThrIleCysAlaGlyTyrCysProThr		
hLH	- -		
		<u>B101/B107/B109</u>	60
hCG	MetThrArgValLeuGlnGlyValLeuProAlaLeuProGlnValValCysAsnTyrArg		
hLH	- Met - - - Ala - - Pro - - - - Thr - -		
		<u>B108/B112</u>	80
hCG	AspValArgPheGluSerIleArgLeuProGlyCysProArgGlyValAsnProValVal		
hLH	- - - - - - - - - - - - - - - Asp - - -		
		<u>B107/B109</u>	100
hCG	SerTyrAlaValAlaLeuSerCysGlnCysAlaLeuCysArgArgSerThrThrAspCys		
hLH	- PhePro - - - Arg - GlyPro - - - Ser - -		
		<u>B111</u>	120
hCG	GlyGlyProLysAspHisProLeuThrCysAspAspProArgPheGlnAspSerSerSer		
hLH	- - - - - - - - - His - GlnLeuSerGlyLeuLeuPhe		
		<u>SCIP</u>	140
hCG	SerLysAlaProProProSerLeuProSerProSerArgLeuProGlyProSerAspThr		
hLH	Leu		
		<u>ProIleLeuProGln</u>	
hCG	ProIleLeuProGln		
hLH			

FIGURE 9



12/16

FIGURE 10



13/16

FIGURE 11

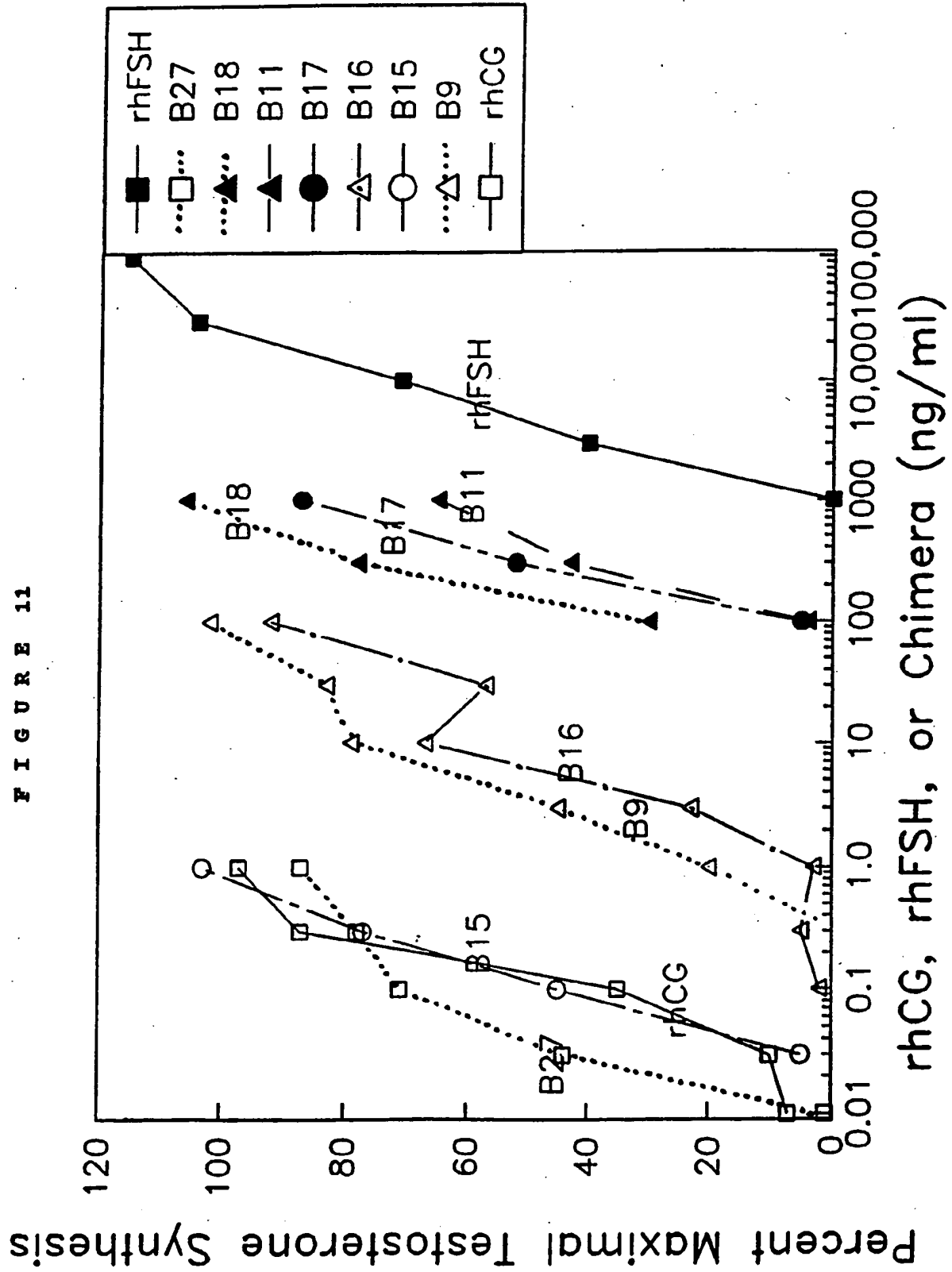
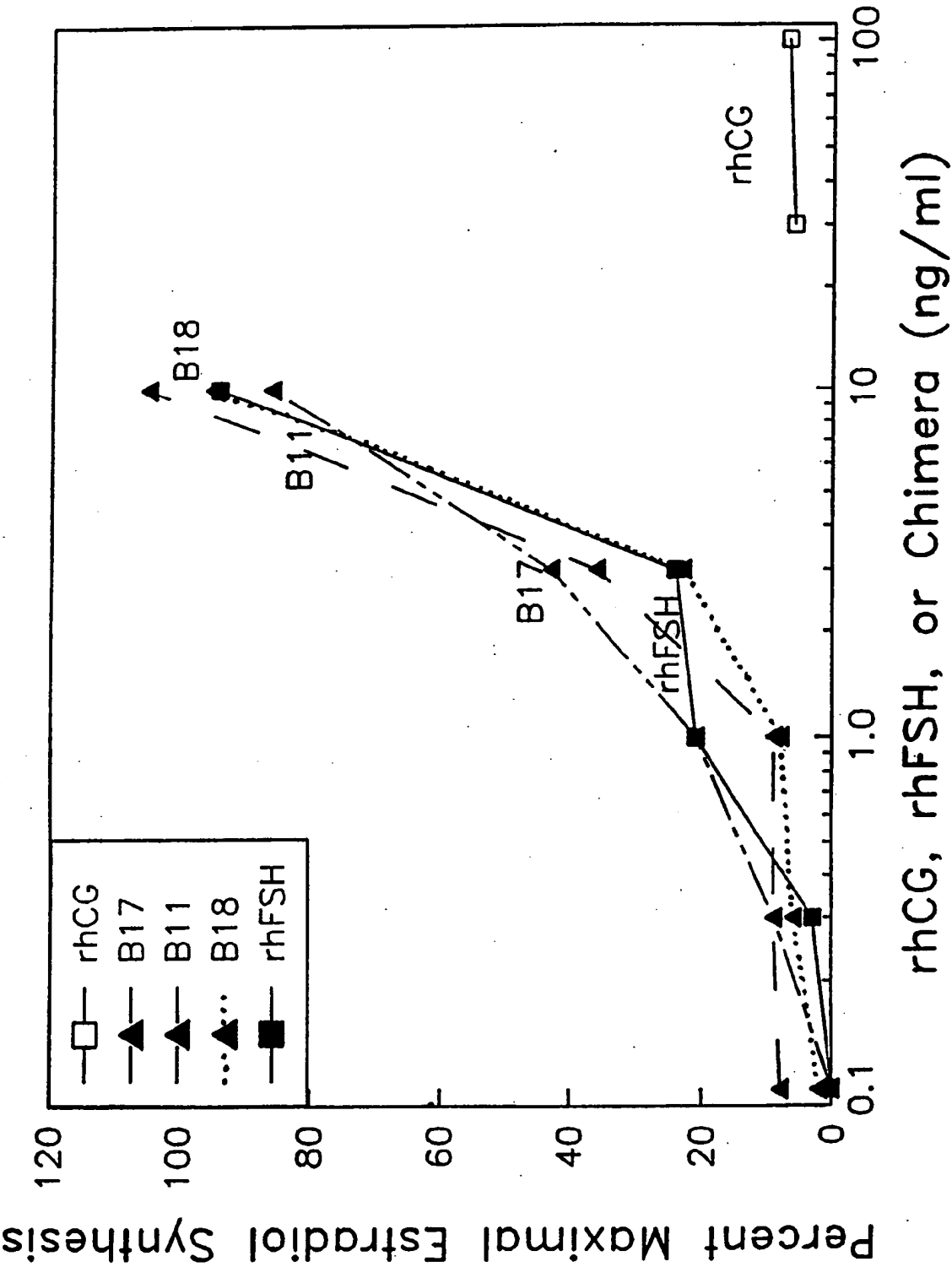
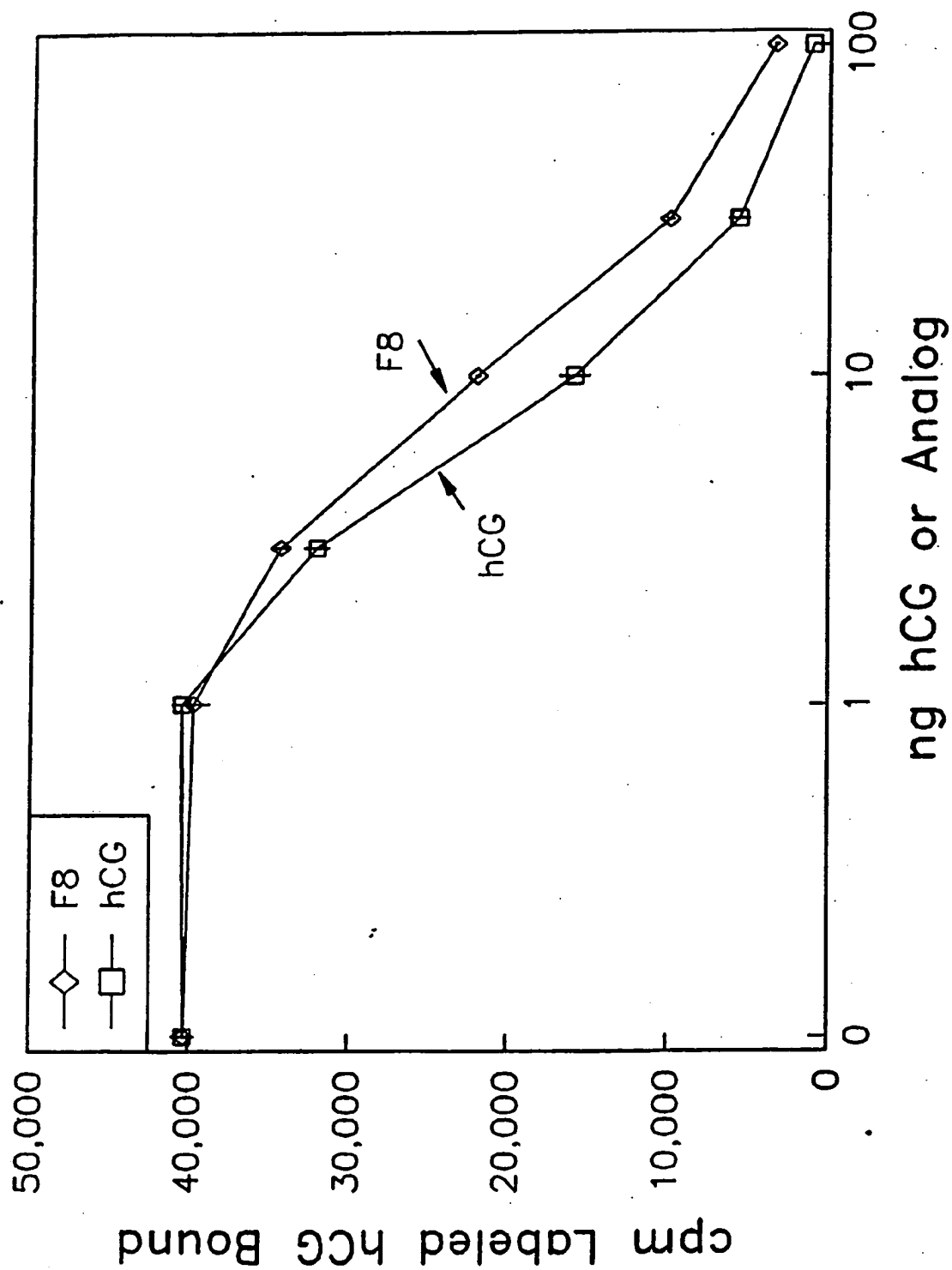


FIGURE 12



15/16

FIGURE 13



**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.